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AN APPLICATION OF THE FROZEN SECTIONING TECHNIC FOR CUTTING SERIAL SECTIONS THRU THE BRAIN

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ABSTRACT.—**The availability of CO_2 ice makes it practical to cut large blocks of cerebral tissue by the freezing method. If the tissue is first treated with 20–30% ethyl alcohol for sufficient time to secure uniform penetration of the alcohol (about 24 hours), formation of hard ice crystals can be controlled and serial sections 25–100 μ thick can be cut with negligible loss. The alcohol can be added to the fixative used for perfusion, or it can be added at any time later in the fixing process, or after fixation is completed. The sections are cemented to the slide and groups of slides are manipulated thru staining processes in glass trays. Ordinary cell and fiber stains give satisfactory results. The method is particularly useful for certain neurophysiological purposes such as defining the location of electrode tracks and lesions and certain types of retrogrades. The Prussian blue test for electrolytically deposited iron can be conveniently applied in conjunction with other stains, to determine the point at which a given action potential response was observed, if steel electrodes are used.**

The determination of the extent of ablations or of the locus of electrode tracks and electrolytic lesions in the brain is a problem encountered in many studies of central nervous functions. For most of these purposes serial sections are either necessary or desirable. Three years use of the frozen section method has led to a procedure which is adequate for many purposes and which can be conducted with considerable economy of time.

The chief obstacle to cutting serial sections of cerebral tissue by the freezing method is the formation of hard and brittle ice crystals which fracture the sections as they are cut. If the tissue is first treated with a 20–30% solution of ethyl alcohol for about 24 hours this difficulty can be avoided. The time required depends on the size of the block; enough time must be allowed to permit uniform penetration of the

alcohol into the tissue. It does not appear to matter how the alcohol solution is used. Any fixation solution to which alcohol can be added may be made up with sufficient alcohol to make a 20–30% solution. Thus the brain can be sectioned, without washing, 12–48 hours after perfusion of the brain. The alcohol can be also added after fixation has begun or is complete; or, after fixation, the brain may be washed overnight and then put into 20–30% alcohol in water for 24 hours and sectioned. In any case, the material to be sectioned should be cut into blocks approximately one centimeter thick. It is sometimes desirable to strip off the pia and usually necessary to remove the extensive vascular tissue at the base of the brain. This can be done most safely the second day of fixation.

When ready for cutting, the tissue is placed on a CO_2 ice freezing block, which has been covered by a piece of wet blotting paper. The solid CO_2 ice is ground in a mortar to a conglomerate of small pieces of ice and snow and the ice chamber is filled with the mixture. For large blocks a little 95% alcohol can be put into the ice chamber with the CO_2 ice to hasten the initial freezing, but the speed of freezing must be increased with caution. Any open places where definite structural discontinuities exist are filled with water. A narrow ice bank must be similarly built up around the edges of thick blocks. It is usually necessary to build up the bank and open spaces by adding water drop by drop, as the block freezes, over a period of 10–15 minutes. As soon as the block is frozen thruout, most of the CO_2 ice is removed from the chamber.

While formation of hard and brittle ice crystals is chiefly controlled by the low concentration alcohol solution, some precautions are necessary in cutting the sections. It is necessary to avoid freezing the block too quickly and to avoid freezing it any harder than necessary. With each stroke of the knife the cut section is removed by means of a camel's hair brush kept wet with 50% alcohol, and it is necessary to paint any ice banks around the block and the colder portions of the block with 50% alcohol. This can be done in a second or two. The appearance of the surface, the force required to push the knife, and the sound the cutting produces are all guides to the estimation of the proper degree of hardness. A little experience teaches the correct judging of this part of the procedure. The essential thing is to keep the surface of the block near the freezing point; the sections are cut from a surface frozen only hard enough to cut cleanly.

A Bausch and Lomb clinical microtome with an 11 cm. paraffin knife is used in the writer's laboratory.[1] The angle of the knife with the block is not critical; it may vary

[1]Credit is due to Mr. J. I. Wexlin of the Bausch and Lomb Company for securing certain modifications of the frozen section microtome and for much helpful advice.

from 20° to 30°. The knife is usually set in a line perpendicular to the direction of motion. Tissue that has been fixed with acetic acid is cut by a razor blade clamped in a device which, in turn, is clamped in the regular knife clamps. There can be no objection to using this for any other type of work as we regularly cut with the knife fixed at right angles to the direction of motion. A freezing block is employed that is somewhat larger than that furnished with the microtome, but of the same general design. This block was cut out of a copper bar, but aluminum is probably to be preferred. There is no reason why other microtomes cannot be used, but the Bausch and Lomb model can be easily altered to give displacements of over 75 μ.

It is practical to mount sections 25–150 μ thick cut thru entire transverse frontal planes, or entire saggital planes of a cat's brain. There does not appear to be any reason why similar sections of larger brains cannot be cut with a correctly designed freezing block, but there has not yet been occasion to do so. As the sections are cut they are placed in order in an enameled pan or glass tray containing water or 30–50% alcohol and left there until the entire block is cut. Shrinkage and consequent curling of the sections can be kept at a minimum only by keeping them in a low concentration of alcohol or in water until they are mounted on the slides. It is helpful to lay glass strips in the pan which mark off the pan into alleys and thus keep the sections in order, in rows. Then they are mounted by placing the section or sections selected for a given slide in a dish of 50% alcohol. They are then floated onto the slide which has been previously coated with Mayer's egg albumin. Each section is smoothed out with a camel's hair brush and the slides are placed on a paraffin mounting tray or in an oven and dried at 40°–50° C. for 15 minutes to an hour.[2] The sections may also be pressed onto the slides with blotting paper, and the drying omitted. In any case, it is desirable to move the sections thru 70% alcohol to 80% alcohol to set the albumin before washing in water. Sections mounted from 50% or 70% alcohol adhere better than those mounted from water. Alkaline solutions should be avoided at each stage in the process of fixing and staining. If an alkaline fixative is used, the block can be made slightly acid by adding a little acetic acid sometime before cutting or the alcohol solution in which the sections are placed can be neutralized by addition of acetic acid. Even the laboratory water (pH 8) will detach the sections if they are washed in it for more than an hour. It is to be noted that silver processes usually involve an alkaline reducer We have found that hydroquinone formalin reducer as recommended by Bodian (1937) does not detach the sections. Amidol at an acid pH has not been tried; (see Davenport, *et al.* 1939).

Gelatin in 2% solution is a satisfactory adhesive if all subsequent procedures are carried out at room temperature.

[2]The author is indebted to Dr. H. W. Magoun for this and several other suggestions incorporated into the procedure.

The mounting is one of the least satisfactory processes in the method, for stray bits of choroid plexus and overlapping edges of cortical gyri are sometimes not seen before the section is stained.

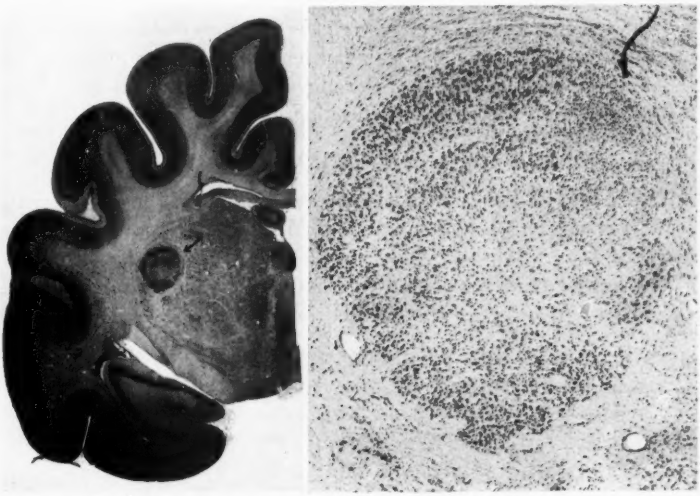
After the series has been mounted the slides are placed in glass trays and dehydrated thru a graded series of alcohol, placed in two changes of clove oil, ether-chloroform or xylene (preference in order named, but we usually use ether-chloroform) for approximately 15 minutes. After this treatment they are run down to water. Thionin (National Aniline and Chemical Company, Inc.), cresyl violet (suggested by Tress and Tress, 1935), toluidin blue (Coleman and Bell Company), and Weil's stains have given satisfactory results. For routine electrode placement sections, it is desirable to keep the differentiation time at a minimum. We have found that this can be done by washing the sections in distilled water 10–30 minutes after the above clearing process, then staining them 15–30 minutes at 50° C. in a saturated solution (less than 0.1%) made up in Sörenson's phosphate buffer calculated for a pH of 5.9. We concede that there is little justification for using this buffer because of the precipitating action of the phosphates. Critical illumination is of considerable aid in using these sections; see Carlson (1935).

There is no difficulty in cutting these sections serially with negligible loss thru a given block. We have not attempted to cut blocks thicker than 12 mm. This method is particularly well adapted for use in conjunction with a Horsley-Clark type of stereotaxic instrument, in which case the animal is perfused with fixatives to be described below, and the brain immediately blocked by partial cuts made by a sharp knife clamped to the needle carriage. These cuts overlap so that the brain is blocked in the plane of the electrode tracks. The animal is then removed from the stereotaxic instrument and the brain excised. When the brain is hardened the cuts are completed, making blocks the faces of which are approximately parallel with the electrode tracks, so that an entire needle track or a considerable portion of it is always seen in one section. For other purposes such as retrograde degeneration experiments it is desirable to outline the blocks similarly in the Horsley-Clark instrument, so that the nuclei desired can be included in a single block. It is, perhaps, worth noting that observation of the surface of blocks as they are cut affords a rather clear view of the gross orientation of electrode tracks.

Any of the usual fixatives should be satisfactory; those used by the writer are 10% formalin, 10% formalin in saline or Ringer's, 10% formalin in Ringer's with enough alcohol to bring its concentration up to 25%. The latter is necessary if sectioning is to be done the following day. It is possible that the fixation is better if the alcohol is not added to the perfusate, and the brain is kept in the formalin for two days, after which the alcohol can be added and the tissue cut a

day or two later. Fixatives causing appreciable swelling of the tissue are obviously undesirable for use in acute experiments in which an area of the calvarium has been removed. For this reason acetic acid fixatives are not entirely desirable for perfusion. We have occasionally used 20% formalin for the purpose of reducing swelling during perfusion with acid mixtures.

This is a very practical means of locating electrode placements, for the brain can be cut and necessary sections stained the day following the experiment.



A photographic reproduction of a transverse section (× 2.3) thru the lateral geniculate nucleus of the cat and a photomicrograph of the lateral geniculate in a contiguous section (× 21.0). The cat was perfused with the alcohol, formalin, Ringer's combination and the fixation was completed in same. The sections were cut at 75 μ, and stained with 0.5% thionin (unbuffered water solution). The retrograde lesion, indicated by arrows, resulted from a small striate cortex lesion made 31 days before the animal was killed.

The Prussian blue test (Adrian and Moruzzi, 1939) for inorganic iron can be introduced in a very convenient way to locate with precision the point at which steel electrodes recorded a given action potential response. Immediately after the observations have been made a current of 10–20 microamperes is passed for 5–10 seconds thru the electrode by putting the positive side of a 3–4.5 volt battery to the electrode connection. The negative pole is connected to a diffuse ground. At the conclusion of the experiment the animal is perfused with a solution consisting of 10% formalin, and 1% potassium ferrocyanide, in Ringer's solution. After the brain is blocked as described above, the animal is removed from the Horsley-Clark in-

strument and the brain is excised and put into the same fixing solution plus enough acetic acid to make a 2% solution, and enough alcohol to make a 20% solution. A day or two later the brain can be sectioned without washing. The electrolytic deposits of iron are a well defined green, usually visible to the naked eye. Thionin in phosphate buffered solution used as described above provides an adequate stain for nuclear groups and contrasts well with the green of the iron; see Talbot (1940). It is also adequate for defining the extents of many types of experimental lesions (Bard, 1940), and is useful for many types of retrograde degeneration experiments. (Figs. 1 and 2.) It is to be noted that thicker sections prepared by this method contain no more tissue than ones 70% as thick cut from blocks imbedded in celloidin or paraffin because of the small amount of shrinkage. This factor is also of advantage in checking electrode spacings.

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COMBINED FIXING, STAINING AND MOUNTING MEDIA

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ABSTRACT.—**A number of non-volatile, water-soluble substances can be added to the usual aceto-carmine fixing fluids. These inert substances do not alter the fixation image and serve as mounting media when the volatile ingredients of the mixture evaporate. Formulae are given for solutions containing dextrin, dextrose, gelatin, pectin, sorbitol, and sucrose. Gum arabic can be incorporated in a formic-acid-carmine fixative. The limiting factor in the use of such mounting media in fixing fluids is the osmotic value they give the solution; with certain precautions, however, they can be used in place of the usual aceto-carmine treatment. The indices of refraction of these media are not as high as those of the natural balsams and the fixation images which the mixtures produce have the characteristic limitations of those secured by the aceto-carmine technic. Some of the natural balsams (Canada balsam, sandarac and Venetion turpentine) can also be incorporated in fixing fluids. These fixatives are able to hold balsam and water in solution together, and, as the volatile components of the mixtures evaporate, the fixed specimens remain in permanent balsam mounts. The addition of carmine to these fluids enables us to fix, stain, dehydrate, clear and mount a specimen in a single operation. These fixatives preserve more details of chromosome structure than the aceto-carmine fluids, but their use is more limited; and they can be substituted for the latter only with certain favorable material, e.g., pollen mother cells of Rhoeo and Tradescantia and salivary gland chromosomes of Chironomus. Some of the newer synthetic resins can be substituted for the natural balsams. Formulae are given for fixatives which contain Venetian turpentine, sandarac, Canada balsam and two synthetic resins.**

Recently there has been a great increase in the use of cytological methods permitting microscopic examination of specimens immediately after fixation. Perhaps the most useful of these procedures has been to macerate or smear the specimens in 45% acetic acid saturated with carmine (Schneider, 1880), thus fixing, staining and mounting the material in a single operation. The addition of a trace of iron greatly intensifies the stain (Belling, 1921, 1926). This acetic-acid-carmine technic has proven especially valuable for investigation of meiotic divisions in pollen mother cells and for examining the giant

chromosomes in the salivary glands of the Diptera. The preparations thus made, however, are not permanent; and further treatment is necessary to preserve them for future reference (McClintock, 1929; Steere, 1931; Buck, 1935; Marshall, 1936; Bridges, 1937; Hillary, 1938, 1939; Burrell, 1939). The addition to the fixing and staining media of various inert substances, which do not alter the fixation image and which serve as mounting media when the more volatile components of the mixture evaporate, greatly facilitates the making of durable mounts. The very simplicity of such a technic gives it a number of practical applications.

These aceto-carmine fixing fluids which contain water-soluble mounting media can be applied successfully to a great many different types of material; in fact, if a few elementary precautions are observed, they can be substituted for the usual aceto-carmine mixtures. This method of making "permanent" mounts, however, has certain inherent limitations and should not be used when it is desired to preserve the finer details of chromosome structure. The water-soluble media have a lower index of refraction than the balsams and, while preparations kept in a dry place have shown no signs of deterioration over a three-year period, they are quickly destroyed if they come into contact with water. The most serious fault, however, is in the fixation image, for many of the finer details of the chromosomes are destroyed by 45% acetic acid. In fact, much of the more careful work of today avoids the acetic acid fixation image by means of a "pre-fixation" technic or by fixing first with a mixture which is less destructive than acetic acid, the aceto-carmine being retained in the technic primarily as a stain (Nebel 1939).

At present the balsams are by far the most satisfactory mounting media. Altho specimens have to be dehydrated and cleared before they can be impregnated with balsam, such preparations are used today almost exclusively. The studies herein reported, however, have shown it to be possible to make fixing fluids in which a balsam can be dissolved directly. The further addition of a dye produces a fluid which enables us to fix, stain, dehydrate, clear and mount the specimen in a single operation and thus simplifies greatly the making of permanent microscopic slides. As these fixatives preserve more detail than does 45% acetic-acid, and as the fixation images can be readily modified in a number of directions, preparations thus made have none of the disadvantages of those mounted in water-soluble substances. While this technic is not suited for preparing all types of material for cytological examination (it cannot be substituted generally for the aceto-carmine technic), it preserves specimens for which

it is fitted about as well as any of the more elaborate procedures.

Several of the newer synthetic resins can also be used as mounting media. They can be incorporated in fixing and staining solutions and treated precisely as the natural balsams. While none of those thus far investigated shows any particular advantages over the natural products, it is possible that some may be synthesized which will have optical properties and solubilities rendering them more satisfactory as mounting media.

I. Water-soluble Mounting Media

General Considerations. The more valuable water-soluble mounting media have been, with few exceptions, mixtures of two or more non-volatile substances. Farrant's solution (gum arabic, glycerin and water) and glycerin jelly (gelatin, glycerin and water) are typical of many such media. At least one component of each mixture must become an amorphous transparent solid on drying; and as these solids are generally too hard and brittle for permanent microscopic preparations, the practice has been to give them the proper consistency by adding to them a certain amount of glycerin. This has introduced the further complication, that when enough glycerin has been added, the fluids acquire such an osmotic value that they plasmolyze many of the more delicate specimens. Even when mixed with rapidly penetrating fixatives the glycerin may cause some shrinkage or prevent that swelling of the nuclear elements which has proved to be such a valuable attribute of many fixation images.

Indeed, the osmotic value of the non-volatile components limits very definitely the amount which can be incorporated in aceto-carmine fluids. On the other hand, these components must have a sufficient volume to occupy the space under the coverglass as the mount dries. Obviously, there will be a greater margin of safety if the substances which serve as mounting media have a relatively low osmotic value per unit volume. It is also necessary that the preparations dry out evenly and this demands that they be hygroscopic enough to pull water out from under the center of the coverglass to the edge where it can evaporate. For example, when gelatin is added to aceto-carmine, it solidifies very quickly around the periphery of the coverglass and seals off the rest of the mount from the air. The result is that the mount remains liquid for several weeks, and the subsequent uneven drying makes it worthless.

There are a number of solids which can be profitably incorporated in the aceto-carmine mixtures to form the basis of a mounting medium. Those investigated by the writer were dextrin, dextrose, sucrose, gelatin, gum arabic and pectin. These acquire a suitable

consistency, however, only when they are combined with some non-volatile water-soluble liquid. The liquids tested were glycerin, sorbitol (a hexa-basic alcohol) and gluconic acid. Glycerin was ultimately discarded in favor of sorbitol[1] for the latter has a larger molecule than glycerin and consequently has a lower osmotic value per unit volume. It also has a higher index of refraction and changes less under varying conditions of humidity. Gluconic acid apparently has no especial advantages.

It should be emphasized that the formulae here given are in no way definitive. They are merely samples of mixtures which have proved useful in mounting specimens of the pollen mother cells of *Rhoeo* and of several species of *Tradescantia*. They should be modified, of course, to suit whatever material is being investigated.

Dextrin. The dextrin should be c.p. and free from starch. The following formula is suitable for the pollen mother cells of *Tradescantia:*

Dextrin	10 g.
Sorbitol	10 cc.
Acetic acid	50 cc.
Water	60 cc.
$Fe(NO_3)_3 \cdot 9H_2O$	0.5 g.
Carmine	to saturation[2]

The dextrin should first be dissolved in the water and the other components added in the order given. It is best to add the carmine after the ferric nitrate has been dissolved. The mixture should be brought to a boil and filtered.

Gelatin. If 10 g. of gelatin are mixed with 100 cc. of Belling's aceto-carmine (the gelatin should be dissolved in the water, the other components added and the whole boiled for 30 seconds and filtered), a liquid mounting medium is secured which automatically becomes sealed as the gelatin solidifies around the periphery of the coverglass. Such preparations dry out very slowly and may remain liquid for several weeks, the gelatin[3] furnishing an excellent, if temporary, seal.

For more lasting preparations the gelatin must be combined with some substance which insures that the entire mount will harden slowly. In the past, glycerin has been used for this purpose, and

[1] A satisfactory brand of sorbitol is sold by the Glyco Products Company, Inc., under the trade name of *Yumidol.*

[2] To saturate this and the following solutions with carmine, it is advisable to add 0.5 g. of the dye to each formula. This is somewhat in excess of the amount that will go into solution; but in the case of the venetian turpentine combination (page 147), it is a good safeguard to have some undissolved carmine at the bottom of the container.

[3] The gelatin is altered, of course, when it is boiled in acetic acid. It is not necessary for our purpose to identify the reactions.

glycerin may be incorporated in the aceto-carmine fluids. It is not recommended, however, for its osmotic value is too great in proportion to its volume. Parenthetically, it may be noted that the gelatin mounting media described below do not liquefy or soften in hot weather, and in this respect they are superior to the well-known glycerin-jelly. They have one defect, however, which need not be serious—small crystals are formed at the periphery. It should be emphasized that the exact proportions of the ingredients in these many-purpose fluids are to be modified to suit each specimen. The following formula may be useful as a point of departure for these necessary modifications:

Gelatin	10 g.
Sorbitol	10 cc.
Acetic acid	50 cc.
Water	60 cc.
$Fe(NO_3)_3 \cdot 9H_2O$	0.5 g.
Carmine	to saturation

Ten grams of sucrose can be substituted for the sorbitol in the above, altho when this is done the mixture should be brought to a boil, but the excess carmine should not be filtered out. The undissolved carmine which remains insures that the solution will remain saturated, for otherwise the color slowly fades. If dextrose is mixed with gelatin, the mounting medium becomes too hard and brittle for practical uses. Gluconic acid and gelatin can be combined as follows:

Gelatin	10 g.
Gluconic acid	15 g.
Acetic acid	45 cc.
Water	55 cc.
$Fe(NO_3)_3 \cdot 9H_2O$	0.5 g.
Carmine	to saturation

When gluconic acid is used, undissolved carmine should likewise remain in the solution. Even then the preparation may fade.

Gum Arabic, Gum Acacia. Gum arabic is soluble in 45% acetic acid but insoluble in stronger concentrations. Consequently, when it is added to aceto-carmine fixatives, the preparations become opaque when they dry in such a way that the water evaporates faster than the acid. On the other hand, the mount remains perfectly transparent if the acid evaporates faster than the water. Opaque mounts are avoided entirely if an acid more volatile than acetic is used. Pianese (1892) described a formic-acid-carmine combination which gives results in many ways more precise than the usual aceto-carmine fixatives, the chromatin being preserved with a sharper outline. As formic acid is also an aldehyde, it must never be mixed with gelatin.

The following is one of the most useful mixtures:

Gum arabic	10 g.
Sorbitol	10 cc.
Formic acid (87%)	41 cc.
Water	65 cc.
$Fe(NO_3)_3 \cdot 9H_2O$	0.5 g.
Carmine	to saturation

If small bubbles appear around the periphery of the coverglass, the amount of sorbitol in the mixture should be increased slightly and the amount of gum arabic decreased. The gum arabic can be most easily incorporated in the mixture if first pulverized in a mortar and dissolved in the water.

Pectin. Pectin[4] forms a gel in the presence of sugar and an acid. However, enough pectin cannot be added to aceto-carmine to serve as a mounting medium; other non-volatile substances must supply the volume. The advantage of using pectin lies in the fact that the preparation becomes solid as soon as it is made, altho it does not become really hard until it dries. There are a number of commercial brands of pectin available, the easiest to use being "Certo." Karo corn syrup (Patrick, 1936) is a satisfactory source of dextrose. The following mixture forms a usable mounting medium.

Certo (pectin)	10 cc.
Karo (dextrose)	10 cc.
Sorbitol	5 cc.
Acetic acid	55 cc.
Water	55 cc.
$Fe(NO_3)_3 \cdot 9H_2O$	0.5 g.
Carmine	to saturation

The mixture should be filtered and the excess carmine removed before the Certo is incorporated.

Directions. Aceto-carmine fixing fluids which contain mounting media are more viscous than those which do not. This introduces a complication in the smear technic, for delicate cells may be distorted in such a liquid by a too vigorous smearing. If pollen mother cells are to be fixed, the whole anther should be covered by the fluid for from 30 to 60 seconds before it is crushed. The cells should be examined immediately after they are extruded from the anther. They should not be shrunken; if they are, the mixture should be diluted with Belling's aceto-carmine. Very delicate cells can be more safely smeared in Belling's solution and then mounted in the

[4]For a detailed description of the pectin gels, see the contributions of L. W. Tarr, L. L. Baker and P. B. Myers in the Delaware Agr. Exp. Sta. Bulls., 136–187. 1924–1934.

mixture. It is important, however, not to mistake the absence of the customary swelling of the cells for a shrinkage. This swelling, which is such a valuable attribute of the aceto-carmine technic, occurs only when the preparation is flamed. The mount should be heated almost to the boiling point to secure the optimum swelling and staining. The amount of solution to be used depends upon the size of the cover glass and the desired thickness of the finished preparation; three or four drops of the solution are generally sufficient. Flexible number 0 cover glasses adhere to the media better than do the thicker types. If some of the medium is extruded around the edge of the cover glass, it should not be removed for it greatly strengthens the mount. For the most satisfactory results, it is necessary to modify and adapt the technic for each type of specimen to be mounted.

II. Fat-soluble Mounting Media

General considerations. To the best of the writer's knowledge, the technic of dissolving a balsam in a fixative has not been used hitherto. Under the circumstances it may be well to consider the principles involved in such a procedure in addition to the usual *ad hoc* directions and detailed recipes. Dissolving a balsam in a non-aqueous fixing fluid which gives an acceptable image offers no real difficulty. The only additional requirement is that the mixture be able to dissolve the water which is in the specimen itself without losing the balsam by precipitation. The addition of a reliable stain, however, introduces complications; in fact, hematoxylin and auramin were the only dyes found which stain the chromatin differentially when incorporated in these mixtures. Carmine[5] was too insoluble to be used. The intensity of the hematoxylin and auramin staining, however, could not be controlled accurately, and consequently the definition was generally poor. Another disturbing factor, which tended to make the staining still more erratic, was the alteration of the staining properties of the solution on standing.

The addition of water to the fixing fluids resulted in two marked improvements: (1) it enabled them to dissolve carmine, a dye easy to control, and (2) it increased greatly the control of the fixation images. As far as chromosome structure is concerned, the image may be altered in any one of a number of directions by changing the proportions of the ingredients; in fact, almost any of the usual fixation

[5]Carmine, of course, can be used in non-aqueous fixatives. In Carnoy's, for example, enough can be dissolved to stain the chromatin faintly. If methyl alcohol be substituted for the ethyl alcohol in the formula, a brilliant stain results. The fixation image, however, has little to recommend it and the mixture is not stable, the carmine being precipitated after a few days.

images are made available. On the other hand, the addition of water introduced another problem: it was necessary to hold the water and balsam together in a single solution, and this involved principles not generally met with in cytology.

Water and an oil can be made miscible by adding to them enough of a third liquid in which they are individually soluble. For example, if 10 cc. of xylene and 10 cc. of water are placed together in a graduate they will separate into two layers. If ethyl alcohol is added slowly, it will be partitioned between the xylene and water until 40 cc. has been added, whereupon it pulls the xylene and water into a single clear solution. In like manner it is necessary, if this technic is to prove practical, to hold both balsam and water in a single solution by reagents which, under these conditions, give a satisfactory fixation image.

The common reagents available in most laboratories of cytology differ greatly in their ability to hold water and an oil in solution simultaneously as is shown in the following table. The amounts are all expressed in round numbers, for the experiments were performed at laboratory temperatures and especially purified reagents were not employed.

TABLE 1. AMOUNT OF REAGENT REQUIRED TO HOLD WATER AND CERTAIN OILS IN SOLUTION SIMULTANEOUSLY

	10 cc. water and 10 cc. xylene	10 cc. water and 10 cc. oleic acid	10 cc. water and 10 cc. clove oil
Methyl Alcohol	90 cc.	80 cc.	70 cc.
Acetic Acid	60 cc.	115 cc.	30 cc.
Dioxan	60 cc.	50 cc.	25 cc.
Acetone	50 cc.	40 cc.	30 cc.
Phenol	50 cc.	35 cc.	25 cc.
Ethyl Alcohol	40 cc.	20 cc.	20 cc.
Tert.-Butyl Alcohol	30 cc.	30 cc.	25 cc.
Propionic Acid	30 cc.	40 cc.	20 cc.
Ethyl Alcohol —2 pts. n-Butyl Alcohol—1 pt.	30 cc.	25 cc.	20 cc.
Phenol —1 pt. Acetic Acid —1 pt.	30 cc.	20 cc.	15 cc.
Phenol —11 pts. Propionic Acid— 7 " Acetic Acid — 3 "	21 cc.	15 cc.	19 cc.

The reagents which are more effective in holding xylene and water in solution together are in general more effective in holding oleic acid and clove oil in solution with water. The table reveals, however, some marked exceptions. It is important to note that certain mixtures of the reagents are much more effective than any of the reagents used singly. For example, 60 cc. of acetic acid or 50 cc. of phenol are required to hold the xylene and water in solution, but when they are mixed in equal proportions only 30 cc. of the combination are needed. The mixture of phenol, propionic acid and acetic acid is the most effective yet found.[6] This mixture can be used as the basis of a fixing fluid.

Fat-soluble mounting media differ greatly in their tolerance for water. Thus, gum mastic and gum damar proved so intolerant that they could not be incorporated in any practical fixative. On the other hand, Canada balsam, sandarac, and Venetian turpentine were employed successfully; but inasmuch as Venetian turpentine provides a much greater margin of safety than the other two media, it alone is recommended. Formulae containing Canada balsam and sandarac are included only for completeness.

Venetian Turpentine. The proportion of the several ingredients in the following formula is determined by three considerations: (1) The Venetian turpentine and water must be held in a clear solution which does not become cloudy on drying; (2) a sufficient quantity of carmine must be soluble for staining; and (3) the fixation image must be acceptable.

Venetian turpentine	20 cc.
Phenol	55 cc.
Propionic acid	35 cc.
Acetic acid (glacial)	10 or 15 cc.
Water	25 cc.
$Fe(NO_3)_3 \cdot 9H_2O$	0.5 g.
Carmine	to saturation

To this it is sometimes necessary to add 5 drops of ethylenediamine. This formula can be put together in a graduated cylinder as follows: The propionic acid is first poured in and then the viscous Venetian turpentine is thoroly mixed with it. Next is added the phenol; either as loose crystals or a liquid. [If liquid phenol (88%)

[6] When phenol, propionic acid and acetic acid are added slowly to the xylene and water in the proportions indicated, the mixture of the five liquids emulsifies readily. When barely enough of the reagents has been added to pull the xylene and water into a single layer, the solution is clear in transmitted light, but opalescent in reflected light, indicating that some of the molecules are grouped in colloidal particles. The possibility is suggested that the molecules are definitely oriented with regard to each other and that the fluid possesses a certain structure.

is used, due allowance must be made for the water it contains.] The acetic acid comes next, and then the water. The ferric nitrate should be dissolved before any carmine is added. Under no circumstances should the mixture be heated. It may be filtered after 12 hours, altho some undissolved carmine in the bottom of the container does no harm. Special care should be taken not to allow the fluid to come into contact with the skin.

The proportions of the several ingredients in this formula have been derived empirically. They will doubtless have to be altered to suit the different specimens to which it is applied. Under the circumstances, it may be well to indicate briefly the role of each ingredient and to show the effects of changing its concentration. As the part played by Venetian turpentine is relatively passive, its concentration may be held constant and the variations limited to the other components.

The phenol ties the Venetian turpentine into the fluid, altho propionic acid may be substituted for much of it without causing any precipitation. A decrease in the amount of phenol decreases slightly the solubility of the carmine. The limiting factor in regard to phenol is the fixation image, as this is controlled by a very complex relationship between the phenol, the acids and water.

The propionic acid may be safely held constant. It has the same fixing properties as acetic acid, but it holds the fat-soluble components in solution better. It can take the place of some of the phenol, but nothing is gained by making the substitution.

It is perhaps more convenient to vary the acetic acid than any other component. This acid ties the water into the mixture. When the fluid is first made, 10 cc. of the acid is sufficient. On standing, however, the fluid alters somewhat and consequently a slight change occurs in its fixing properties. The original fixing properties can be restored by the addition of 5 cc. of acetic acid[7]. It may be noted parenthetically that the fixation image of most of the meiotic phases remains constant even if the proportions of the several ingredients are altered as much as 50%. The first meiotic metaphase, however, is very sensitive to such changes and it is possible, by keeping the phenol and water constant and varying the acetic acid, to secure a number of the more popular images. The proportions given here preserve the chromatic elements of the chromosomes of *Tradescantia* as spiralized chromonemata. This choice of fixation image is, of

[7]Even after standing six months the characteristic image of the fresh solution can be restored by adding another 5 cc. of acetic acid.

course, somewhat arbitrary, altho when this image is chosen, homologous chromosomes in different cells show less individual variation than they do when they are fixed as parallel threads, or, at the other extreme, as swollen, heavily staining bodies with a central row of vacuoles.

The water controls the amount of carmine which the solution dissolves. The valuable swelling of the nuclear elements characteristic of the aceto-carmine technic also depends upon the presence of of water.

The amount of iron mordant to be incorporated must be accurately determined. The best results for routine use are obtained by using either 0.5 g. of ferric nitrate or 0.2 g. of ferric chloride. If less iron is used, the stain is not sufficiently intense; if more is used, the iron tends to "salt out" the water so that the mount may become opaque on standing. This separation of water from the other ingredients as the mount hardens can be prevented by adding ethylene-diamine, which, by forming a complex cation with the iron, prevents the "salting out" effect. Thus, when it is desired to increase the staining capacity of the fluid, more iron can be included if at the same time ethylene-diamine is added. The quantities of each will have to be determined by experiments. Too much ethylene-diamine decreases the staining capacity of the mixture; too little allows it to cloud.

Canada balsam and sandarac. The following formula has given results comparable to those of the Venetian turpentine mixture. It is less tolerant of water, however, and consequently has a more limited usefulness.

Canada balsam or sandarac	10 g.
Oleic acid	10 cc.
Phenol	65 cc.
Propionic acid	40 cc.
Acetic acid (glacial)	15 cc.
Water	20 cc.
$Fe(NO_3)_3 \cdot 9H_2O$	0.5 g.
Carmine	to saturation

Directions and precautions. The limiting factor in the use of fixatives which contain a balsam in solution is the water which is in the specimen itself. If too much water is introduced, the preparation clouds on drying. The remedy is either to fix a smaller specimen or use more of the fixative. The amount of fixative also depends upon the thickness of the preparation and the size of the cover glass. In making smears of the anthers of *Tradescantia*, three drops are enough for a 22 × 40 mm. cover glass and four drops for one 24 × 50 mm.

The following method has been successful with pollen mother cells of this species. The anthers are placed uncrushed on a *dry* slide and covered by one drop of the fixative, which is allowed to penetrate for from 30–60 seconds. The anthers are then smeared and two more drops of the fixative added. After standing a few seconds, the preparation is ready for the cover glass. The preparation should *not* be flamed. The characteristic swelling occurs immediately on fixation, altho the stain becomes slightly more intense during the first 24 hours. This slide is permanent as soon as it dries. No special precautions are necessary in the dry air of a steam heated laboratory; the slide may simply be left to harden on a table. On a humid day in summer, however, or in the moist air of a seaside laboratory, the water evaporates relatively slowly, while the acids and phenol evaporate at their usual rate. If the water evaporates more slowly than the other volatile components, the mount becomes opaque. This can be prevented by drying the slide on a hot plate or in a desiccator. It can even be dried in a paraffin oven (60° C.) without injury.

Limitations. These fluids have been used successfully in making smear preparations of small root tips of *Zea Mays* and in preparing permanent mounts of salivary gland chromosomes of *Chironomus.* They are suited, of course, for fixing, staining and mounting the pollen mother cells of many different plants. For some material, however, they are quite unsuited; e.g., the pollen mother cells of *Oenothera* and *Gossypium.* Apparently any specimen which contains tannic acid, or any other compound which forms a precipitate with the iron mordant, cannot be treated successfully with this technic.

III. Synthetic Resins

Recent advances in the synthesis of new resins promise to provide mounting media superior to any now in use. Both water-soluble and fat-soluble resins are available, and it is possible that some of these, which the writer has not investigated, have just the properties which are needed. None of the water-soluble resins which were tested, however, proved stable in the concentration of acetic acid necessary for the aceto-carmine technic. On the other hand, two of the fat-soluble resins were stable in the mixture of phenol, propionic acid, and acetic acid, and as they were slightly more tolerant of water than Venetian turpentine, they might be preferred to the latter under certain conditions. This advantage, however, is slight, and as Venetian turpentine has a higher index of refraction and adheres better to glass, it should be used in preference to these where possible.

Duraplex V–240.[8] The formula for the mixture containing this resin is as follows:

Duraplex V–240	30 cc.
Phenol	60 cc.
Propionic acid	40 cc.
Acetic acid	15 cc.
Water	30 cc.
$Fe(NO_3)_3 \cdot 9H_2O$	0.5 g.
Carmine	to saturation

This is to be used precisely as the Venetian turpentine mixture.

NY–83–8. This synthetic resin is handled more easily as a 70% solution in methanol. It does not adhere to glass as well as Venetian turpentine. The following formula can be used:

Ny–83–8 (70% in methyl alcohol)	30 cc.
Phenol	50 cc.
Propionic acid	35 cc.
Acetic acid	20 cc.
Water	30 cc.
$Fe(NO_3)_3 \cdot 9H_2O$	0.5 g.
Carmine	to saturation

Lucite. Lucite is the trade name of a plastic made by E. I. du Pont De Nemours & Co. It is soluble in the mixture of phenol, propionic acid, acetic acid and water; and as it has a high index of refraction and adheres well to glass, it forms an excellent mounting medium. Two of its characteristics, however, render it unsuited for this technic. Mixtures which contain as little as 8% Lucite are so viscous that unless great care is taken, the cells of the specimen will be distorted when they are smeared in it. A more serious defect appears as the mount hardens. Fine droplets of water form (often temporarily) and any cells in contact with them become badly shrunken. The related mounting medium, isobutyl methacrylate, is even less tolerant of water than Lucite itself.

All grades of Lucite are soluble in the fixing mixture. Soft crystals (H7500) with type "D" plasticiser are easiest to handle. While passable slides can be made with the following recipe, it is not recommended.

Lucite	10 g.
Clove oil	10 cc.
Phenol	65 cc.
Propionic acid	45 cc.
Acetic acid	20 cc.
Water	20 cc.
$Fe(NO_3)_3 \cdot 9H_2O$	0.5 g.
Carmine	to saturation

[8]This is the trade name of a resin produced by *The Resinous Products and Chemical Co., Inc.*, of Philadelphia. The other resin designated Ny-83-8 is also their product.

Conclusions

Two types of mounting media which can be incorporated in fixing and staining solutions have been described. Those which are water-soluble can be added to the usual aceto-carmine fluids (gum arabic in formic-acid-carmine) and can be used wherever the usual aceto-carmine methods are suitable. The limiting factor is the osmotic value which the media give the solutions, so that sometimes special precautions are necessary. The swelling which is such a valuable consequence of acetic acid fixatives can generally be secured by heating the preparations to the boiling point. Slides made by this method have been kept in a dry place for three years and have shown no signs of disintegration. The fixation image has the characteristic limitations of that given by the aceto-carmine fluids.

Fat-soluble mounting media (Canada balsam, sandarac, Venetian turpentine) can be incorporated in a fixing and staining solution composed of phenol, propionic acid, acetic acid, and water. The fixation image given by this solution can be controlled more precisely than that of the aceto-carmine fluids. The limiting factor in the use of these fixatives is the water in the specimen itself. Sometimes special precautions are necessary in drying the preparations, but once dry they are as permanent as the usual balsam mounts. This technic cannot be used with material which contains tannin or other substances which precipitate the iron mordant. These fluids have been successfully employed on the pollen mother cells of *Rhoeo* and several species of *Tradescantia* and on the salivary gland chromosomes of *Chironomus*. They are not suited for preparing the pollen mother cells of *Gossypium* or *Oenothera*.

Synthetic resins can be incorporated in the mixture of phenol, propionic acid and acetic acid. One of them, Duraplex V–240, has a greater tolerance for water than has Venetian turpentine, altho it does not adhere to glass as well. Some of the newer resins will probably form better mounting media than any of the natural products now in use.

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THE SELECTIVE STAINING OF RED BLOOD CELLS

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ABSTRACT.—**A method for the selective staining of red blood cells is described. Material is fixed in 10% neutral formalin in .85% NaCl and imbedded in paraffin or celloidin. Sections 6–10 μ are stained 1–5 minutes in chromotrope 2R. Basophilic and the less strongly acidophilic elements are decolorized with 5% phosphotungstic acid in 95% ethyl alcohol. Red blood cells and other strongly acidophilic elements that may be present in the preparation retain the chromotrope 2R. A counterstain of methyl blue may be used for staining the decolorized basophilic elements. As a result, erythrocytes are stained red by the chromotrope 2R, and basophilic elements blue, by the methyl blue. Less strongly acidophilic elements, having little affinity for either primary or secondary dye, are colorless or gray.**

In a previous paper[1] the writer described a staining method for the differentiation of the acidophilic elements of tissue into two groups. Sections, appropriately fixed, were stained with erythrosin or other substituted fluorescein dye and differentiated in phosphotungstic acid. Following immersion of the sections in absolute ethyl alcohol, all tissue elements were decolorized with the exception of those strongly acidophilic. This group included erythrocytes, eosinophil granules, certain secretion granules, alpha cells of the pituitary, keratinized epithelia and Russell's bodies. The decolorized basophilic elements, collagen, reticulum, cartilage, bone, mucin, beta cells of the pituitary and basophil granules, were counterstained with anilin blue or light green. Less strongly acidophilic elements, including smooth muscle, striated muscle and the ground substance of most epithelia were characterized by their slight affinity for either primary or secondary dyes.

Since the publication of this paper, the technic has been simplified and a dye, chromotrope 2R, has been found superior to the fluorescein dyes for this procedure. The present technic is especially designed for the selective staining of red blood cells. The application of chromotrope 2R for staining the other above mentioned strongly acidophilic elements is under further investigation at this time.

[1]Crossmon, G. C. 1939. Separation of the acidophilic elements of the tissues into two groups. Anat. Rec., **73**, 163–70.

The procedure is as follows:

1. Fix in 10% neutral formalin in 0.85% NaCl for 24–48 hours. Commercial formalin may be neutralized by the addition of marble chips a few days prior to fixation. Other fixatives may be employed but are not recommended for the preservation of red blood cells. Following fixation, material is washed in several changes of 70% ethyl alcohol to remove excess formalin.

2. Dehydrate completely, imbed in paraffin or celloidin, cut and mount as usual. It is important that sections be thin; with increasing thickness, decolorization in step 5 becomes more difficult. Six to 10 μ is suggested.

3. Stain sections in chromotrope 2R.

Chromotrope 2R (Grübler or National Aniline Co.)	0.25 g.
Distilled water	100 cc.
Glacial acetic acid	1 cc.

A suggested staining time is 1–5 minutes.

4. Rinse sections in 4 or 5 changes of distilled water. The last change of distilled water should show no trace of dye.

5. Transfer sections to 5% phosphotungstic acid in 95% ethyl alcohol. Agitate the slides occasionally while in this reagent. Preparations are left in phosphotungstic alcohol until as observed under the microscope all tissue elements are decolorized with the exception of red blood cells. The time varies with the thickness of the sections and the thoroness of the distilled water rinse in step 4. Basophilic elements are readily decolorized. Less strongly acidophilic elements, especially striated muscle, may require as long as 2–3 hours.

The specificity of the stain has been tested by leaving sections in phosphotungstic alcohol for a period of 36 hours without the slightest decolorization of red blood cells.

6. Transfer to methyl blue.

Methyl blue (National Aniline Co.)	0.5 g.
Distilled water	100 cc.
Glacial acetic acid	1 cc.

A suggested staining time is 2–5 minutes.

7. Rinse in distilled water.

8. Transfer to 2% glacial acetic acid for approximately 15–60 seconds. Sections should be examined microscopically at this time. As a result of this differentiation, erythrocytes should be red, less strongly acidophilic elements colorless or gray, and basophilic elements blue.

9. Pass thru 2 or 3 changes of absolute ethyl alcohol followed by 3 changes of xylol. Mount in balsam.

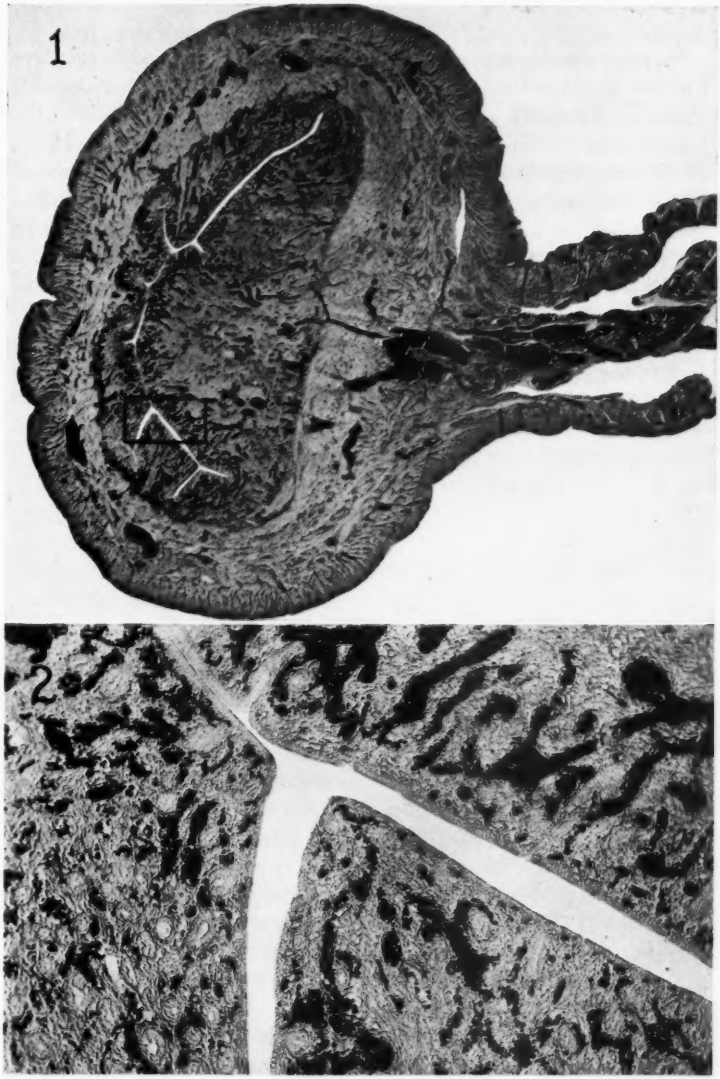

Figure 1. Photomicrograph of uterus of rabbit. Chromotrope 2R and methyl blue. × 10.

Figure 2. Photomicrograph of the area indicated within the rectangle in Figure 1. × 100.

Results and Discussion

Staining results are as given in step 8. The method is selective for red blood cells but not specific. Other strongly acidophilic elements, if present, retain the chromotrope 2R. If it is desired, the technic may be simplified by omitting the counterstain of methyl blue. In this case erythrocytes are stained red on a colorless background of the less strongly acidophilic and basophilic elements. A nuclear stain of freshly prepared Weigert's iron hematoxylin may be used as a preliminary to staining with chromotrope 2R.

Altho thick sections are not suggested, the writer has decolorized celloidin sections up to 25 μ. Such preparations are very striking and are suggestive of carmine-injected material for the demonstration of the circulation.

ACID FUCHSIN AS A CONNECTIVE TISSUE STAIN AFTER PHOSPHOMOLYBDOTUNGSTIC MORDANTING

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ABSTRACT.—**Certain acid fuchsins stain connective tissue deep red after phosphomolybdotungstic mordanting in a modified Masson procedure, others are entirely unsatisfactory for this purpose. Spectrophotometric examination gives no reliable criteria for separation of acid fuchsins satisfactory for this purpose from unsatisfactory ones. Sulphonation of basic fuchsin with 3.5 to 4 parts of 25–30% fuming H_2SO_4 to 1 part of dye gives a satisfactory product at temperatures as low as 65 to 70° C. in 30 minutes, while use of 5 to 7.5 parts of acid at this and at higher and lower temperatures gives unsatisfactory products. Satisfactory products may be produced with 15% fuming H_2SO_4 in similar quantities, and even with concentrated H_2SO_4, but some unconverted basic fuchsin remains with both and, with the latter, lower quantities give unsatisfactory products. Brief chemical studies indicate that oversulphonation may occur in the manufacture of acid fuchsin and that this is just as deleterious as undersulphonation.**

In the course of recent studies on trichrome staining (Lillie, 1938), it was noted that acid fuchsin (C. I. No. 692, lot NR–10) repeatedly failed to give a red connective tissue fiber stain after phosphomolybdic acid mordanting in a modified Masson technic. In this, acid fuchsin appeared to differ from the other commonly used diamino- and triamino-triphenylmethane sulphonic acid dyes such as light green SF (C. I. No. 670), fast green FCF, methyl blue[1] (C. I. No. 706), and anilin blue (C. I. No. 707). This discrepancy was quite disturbing from a theoretical point of view. Another old sample from Eimer and Amend also consistently failed to stain connective tissue.

Later two fresh lots of acid fuchsin, NR–11 and CR–6, were tested as fiber stains as a matter of routine confirmation of the previous results. Both of these samples repeatedly gave excellent red connective tissue staining by the following technic: Sections were first mordanted 1 minute in saturated alcoholic picric acid, washed 3 minutes in running water, stained 6 minutes in Weigert's acid iron chloride hematoxylin, rinsed in water, stained 4 minutes in 1% solution of naphthol green B (C. I. No. 5) in 1% acetic acid, rinsed in water, mordanted 1 minute in equal parts of 5% solutions of phos-

[1]See Lillie (1940).

phomolybdic and phosphotungstic acids, passed directly into a 2.5% solution in 2.5% acetic acid of the acid fuchsin being tested and stained 4 minutes, differentiated 1 minute in 1% aqueous acetic acid, passed thru alcohol, acetone, acetone and xylene aa, and xylene into salicylic acid balsam.

All four samples gave satisfactory connective tissue staining when mixed with picric acid by the van Gieson method.

The possibility was considered that a predominance of rosanilin or pararosanilin sulphonic acids might be responsible. To rule this out, 10 g. lots of an old Grübler sample of rosanilin sulphate, and of a 1936–7 sample of new fuchsin from Hartman Leddon Company, were sulphonated with 50 g. 25% fuming H_2SO_4 holding for 1 hour at 80–85° C. Both of the synthesized dyes gave good connective tissue staining.

Next the experiments of Scanlan, French and Holmes (1927) were repeated in part to ascertain whether the sulphonation conditions necessary to produce an acid fuchsin suitable for collagen staining after phosphomolybdotungstic mordanting are the same as those found best by them for acid fuchsins for van Gieson staining.

Our sulphonation technic is given here:

The fuming H_2SO_4 is weighed into a 150 cc. wide mouthed flask of general spherical form with flat bottom. The flask is clamped by its neck over a water or paraffin bath and a mechanical stirrer rigged in it. The bath is preheated to the desired lower temperature limit. Ten grams of finely ground basic fuchsin are then added gradually to the acid with the mechanical stirrer operating, avoiding boiling by slow addition of the dye. Then the sulphonation flask is immersed in the preheated bath and stirring continued for the designated length of time. When sulphonation is complete, a drop gives a clear red solution in water, which is decolorized by adding NaOH. Then the acid solution is poured into 350 cc. of cold water in a 2 liter beaker, and the flask is rinsed with 3 successive 50 cc. rinses of cold water into the same beaker. The diluted acid solution is then heated to boiling and neutralized with an amount of $CaCO_3$ slightly exceeding the quantity theoretically required by the amount of fuming H_2SO_4 used. For example, 40 g. of fuming sulphuric acid (30% free SO_3) requires approximately 43.6 g. $CaCO_3$; we used 45 g. The $CaCO_3$ is added gradually with constant stirring; the hot thick mixture is immediately poured into a large Buchner funnel and filtered into a vacuum flask. The large beaker is rinsed with successive washings to make a total of 500 cc. of boiling water and the washings are poured thru the precipitate. To convert the calcium salt to the sodium salt of acid fuchsin, 14 g. sodium bicarbonate is added, the solution is heated to boiling, set aside to cool over night, and the precipitate of $CaCO_3$ is filtered out. The solution is neutralized with 12–18 cc. of approximately 6 *N* HCl, testing with congo red paper. The deep red dye solution is evaporated to dryness over a steam bath. The yield is about 20 g. when 10 g. fuchsin and 40 g. fuming H_2SO_4 are used.

Ten-gram samples of a single lot of basic fuchsin were sulphonated for 15 minutes after the indicated temperature was reached, using at

each temperature 40 and 75 g. quantities of fuming H_2SO_4 containing about 25% free SO_3. The temperature ranges were 50–55° C., 65–70°, 80–85°, 95–100°, 110–115°, and 125–130°. Acid fuchsins NR–10 and NR–11 were used as negative and positive staining controls. The lots sulphonated with 40 g. fuming H_2SO_4 for 15 minutes at temperatures of 95–100° C. or higher were quite good, as was that sulphonated at 50–55° C. with 75 g. acid. The larger quantity of acid gave worthless products at 80–85° or higher temperatures, while with the 40 g. quantity of acid the lots prepared at the lower temperatures were all fairly good.

Following these tests, 10 g. lots of another batch of basic fuchsin were sulphonated with 40 and 50 g. quantities of 30% fuming H_2SO_4 at 65–70° C., holding for 15 and 30 minutes and one and two hours after the indicated temperatures were reached, making a total of 8 lots. Acid fuchsins NR–11 and E & A were used as positive and negative controls. This series indicated that the use of 50 g. of 30% fuming H_2SO_4 gave an inferior product and that with longer holding time, the product was poorer. With 40 g. acid the product was excellent and the optimum time appeared to be about 30 minutes. Consequently a larger batch was made with the same proportions, time and temperature. The product was excellent and colorimetrically apparently of higher dye content than any of the commercial samples.

To determine further the limitations on the proper quantities of basic fuchsin and acid for satisfactory sulphonation, another series of sulphonations was performed using the same temperature of 65°–70° C. and 30 minute time interval as previously.

In this experiment 12 g. portions of basic fuchsin were combined respectively with 40, 35, 30 and 25 g. portions of fuming H_2SO_4 containing 30% free SO_3; with 41.7, 36.2, 31.0 and 26.0 g. of a mixture of equal parts of the above fuming H_2SO_4 and of ordinary concentrated sulphuric acids; and with 42.5, 37.4, 32.0 and 26.7 g. of concentrated H_2SO_4.

The processes using the mixture of sulphuric and fuming sulphuric acids, and concentrated sulphuric acid alone yielded, at the stage of treatment with sodium bicarbonate, color precipitates which were soluble in hot water and in HCl and which reprecipitated quantitatively with picric acid solution. These precipitates appear to be unaltered basic fuchsin.

The final products were tested as usual in the Masson technic. Satisfactory products were obtained from sulphonation with fuming sulphuric acid using 40, 35 and 25 g. quantities, from the mixture of fuming and concentrated sulphuric acids in all quantities tried, and

from concentrated sulphuric acid in 42.5 and 37.4 g. quantities, each to 10 g. of basic fuchsin. The products were unsatisfactory with 30 g. fuming H_2SO_4 and with 32 and 26.7 g. concentrated H_2SO_4. (While H_2SO_4 containing only 15% free SO_3 appears to yield satisfactory acid fuchsins, the yield is reduced and some fuchsin escapes sulphonation.)

TABLE 1. COMPARISON OF VARIOUS ACID FUCHSINS IN THE VAN GIESON TECHNIC*

Sample Designation	Amount of acid fuchsin per 100 cc. picric				Rating by Masson Method
	25 mg.	50 mg.	100 mg.	200 mg.	
CR-6	+	++	+++	++	+++
NR-11	+	++	+++	++	++
NR-10	±	+	++	+	-
E & A	-	±	+	+	-
75-125-1	-	∓	±	+	+
75-110-1	∓	±	+	+	∓
75-95-1	-	∓	±	+	∓
75-80-1	±	±	+	++	∓
75-65-1	∓	±	±-+	+	+
75-50-1	±	+	++	++	+-++
40-125-1	∓	±	+	+-++	+-++
40-110-1	±	+	+	++	+-++
40-95-1	±	+	++	++	+-++
40-80-1	±	+	+	++	+
40-65-1	±	±-+	+	++	+
40-50-1	±	±	±-+	+	+
100/25-65-30	+	++	+++	++	+++

*The symbols in this table indicate:
+++ Excellent, ++ Very good, + Good, ± Fair, ∓ Poor, - No collagen staining.

As a measure of further control, many of these acid fuchsins were tested also by the van Gieson technic using various proportions of acid fuchsin as shown in Table 1. Fairly good correlation is shown but the van Gieson method does not give the sharp failures that become evident when the Masson method is applied.

Spectrophotometric examination of two samples CR–6 and NR–10, the one giving bright red connective tissue in the Masson technic, the other not, showed absorption maxima at 540 to 550 mμ in both.

TABLE 2. TRANSMISSION OF LIGHT BY ACID FUCHSINS CR–6 AND NR–10*

Wave Length in mμ	500	520	530	535	540	545	550	555	560	570	580	590	600	620	660	700	
CR–6	75	65	58	53	49	50	50	46	65		95		100	100	100	100	% Transmission
NR–10	75	65	56	60	49	50	49	54	60	75	95	100	100	100	100	100	% Transmission

*Note. Acknowledgment is made to Dr. M. I. Smith for these two examinations.

Believing that further spectrophotometric examinations might be of value, a number of spectrophotometrograms were prepared with the recording spectrophotometrograph of the Washington Biophysical Society and measured in detail. Table 3 gives a summary of these measurements. Characterizing NR–11, CR–6, 40–65–15, 40–65–30 and 100/25–65–30 as satisfactory acid fuchsins from the staining tests, and E & A, 75–125–1, 75–110–1 and 50–65–2 as unsatisfactory, no spectrophotometric character was found which would clearly separate these groups.

The comparatively high absorption percentage given by lot 50–65–2 (*50* g. fuming H_2SO_4 to 10 g. fuchsin, sulphonated at *65*–70° C. for *2* hours) at its absorption maximum when in aqueous solution at 5 γ per cc. indicates that this unsatisfactory sample has a comparatively high dye content. Hence it would seem that low dye content alone was not the reason for the poor staining. Doubling the acid fuchsin content of the 2.5% acetic acid solution used after the phosphomolybdotungstic mordant, while it did slightly improve the connective tissue staining, did not render it satisfactory. The E & A sample, the 75–125–1 and the 50–65–2 lots were thus tested. Except for CR–6, all satisfactory samples show a ratio of the absorption percentage at 530 mμ to that at 560 of 1.1 to 1 or higher, *but* on direct comparison of the stains CR–6 is better than NR–11. The median of

TABLE 3. VARIOUS SPECTROPHOTOMETRIC CONSTANTS OF ACID FUCHSINS

Sample	% Ab. 530 / % Ab. 560	% Ab. 520 / % Ab. 570	% Ab. at max, with 5γ per cc	λ max. Ab. %	λ 95% Ab.	Median λ 95% Ab.
Satisfactory NR–11	1.20	1.91	27.7	542–544	538–550	544
CR–6a	.917	1.15	35.9	547–548	542–556	549
CR–6b	.982	1.29	34.2	547–550	541–553	547
40–65–15	1.24	1.80	33.7	541–543	536–550	543
40–65–30	1.155	1.78	44.3	545	537–550	543.5
$\frac{100}{25}$–65–30	1.11	1.57	45.6	545–548	539–553	546
Unsatisfactory E & A a	.97	1.115	17.0	549–550	536–557	546.5
E & A b	1.00	1.07	27.7 at 10 γ	548–549	531–559	545
75–110–1	.98	1.37	17.2 at 20 γ	545	544–550	547
75–125–1	.923	1.06	18.2 at 20 γ	549–550	544–553	548.5
50–65–2	1.017	1.54	44.0	547	540–553	546.5
NR–10 (Visual)	1.10	1.40	About same as CR–6	545, 550	.....	.....

the zone showing over 95% of the maximum absorption percentage was between 545 and 549 with all the unsatisfactory samples, while the satisfactory samples ranged from 543 to 549, with 2 of the best at 546 and 548, the best and the poorest of the satisfactory samples at 543.5 and 544.

Chemical analyses of 5 samples were made by Senior Chemist Elias Elvove for nitrogen, total sulphur and free sulphate, sodium, calcium, magnesium and chlorine. After deducting calcium and magnesium, free sulphate and chlorine and enough sodium to satisfy the excess of anions over that required by the calcium and magnesium

and dividing the residues by the atomic weights, the following atomic proportions were obtained. (Table 4).

TABLE 4. ATOMIC PROPORTIONS OF N, S, AND Na IN ACID FUCHSINS

Sample	CR–6	100/25–65–30	75–125–1	50–65–2	E & A
N	3.00	3.00	3.00	3.00	3.00
S	3.36	2.52	5.80	3.54	1.50
Na	0.97	2.25	6.60	1.78	2.90

These results, while crude, indicate that the satisfactory samples 100/25–65–30 and CR–6 contain 2 to 3 sulphonic acid groups, that the marginal sample 50–65–2 probably contains 3 to 4 and that the 2 unsatisfactory samples 75–125–1 and E & A are markedly over-sulphonated and under-sulphonated respectively.

CONCLUSIONS

Acid fuchsins may be a satisfactory collagen stain after phosphomolybdic-phosphotungstic mordanting. If sulphonated with an excess of fuming H_2SO_4 or at too high a temperature, the product is unsatisfactory, probably on account of over-sulphonation. Too low temperature or insufficient amount and concentration of SO_3 also yield unsatisfactory products, probably on account of undersulphonation. The absorption spectra of satisfactory and unsatisfactory samples are similar.

ACKNOWLEDGMENTS

To Technician Coey E. Jones for aid in the sulphonations, staining of sections and ruling and measuring the spectrophotometrograms.

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SCANLAN, J. T., FRENCH, R. W., and HOLMES, W. C. 1927. Acid fuchsin as a stain—a refinement in manufacture. Stain Techn., **2**, 50.

THE ACETO-CARMINE METHOD FOR FRUIT MATERIAL

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Abstract.—**It is not easy to make good aceto-carmine preparations of plants with small chromosomes at meiosis because the cytoplasm readily takes up the stain and this prevents a sharp differentiation. The staining reaction depends on the composition of the pre-fixative, the duration of fixation, strength of aceto-carmine and amount of iron used. These factors can be varied independently. Since not only species but their varieties differ markedly from one another in their behavior, the best results can be secured only after experiment with individual plants to discover the most suitable combination. Suitable combinations of these factors for some fruit plants are described. In general they demand (1) a weaker solution of aceto-carmine and more iron than has hitherto been used in the aceto-carmine technic, and (2) the introduction of iron and carmine into the pre-fixative. Iron acetate is added to a dilute solution of carmine in glacial acetic acid until the solution assumes a deep red color, without precipitation, and this solution is used as the acetic acid component of an acetic-alcohol pre-fixative. Anthers are colored purple by treatment with this fixative, but since it has only a mordanting effect they need to be smeared and stained in the ordinary way.**

Plants with large chromosomes, such as *Tradescantia*, give excellent results with aceto-carmine; those with small chromosomes give inferior results owing to the readiness with which the cytoplasm takes up the stain and thus prevents adequate differentiation between chromosomes and cytoplasm.

Experience in this laboratory with the treatment of small chromosomes in various fruit species leads to the belief that what is needed is a radical modification of the fixation and mordanting methods used with aceto-carmine. With such modification these methods might be capable of giving as satisfactory results with small chromosomes as with large.

The staining reaction depends on four factors which can be varied independently: (1) constitution of the pre-fixative; (2) duration of fixation and storage; (3) strength of aceto-carmine solution; (4) amount of iron introduced.

Constitution of pre-fixative. Three parts of absolute alcohol to one

of glacial acetic acid is as a rule quite a satisfactory pre-fixative for aceto-carmine. Variation in the proportion of alcohol and acetic acid alters the quality of fixation but does not appreciably change the staining reaction. Poor fixation, however, results in a poor staining reaction.

Improved staining results can be obtained by introducing both carmine and iron into the fixative. Glacial acetic acid is boiled with excess of carmine and filtered after cooling. A saturated solution of iron acetate in glacial acetic acid is added drop by drop until the carmine-acetic solution assumes a deep red color, without precipitation. This carmine-iron-acetic solution is used to make the 3:1 alcohol-acetic pre-fixative. Occasionally an undesirable precipitate forms in this fixative, either at once or some time after the anthers have been put in. This occurs when the original carmine-acetic solution is too concentrated. The procedure to adopt is to test small quantities of the solution for precipitation after diluting with various amounts of glacial acetic acid.

Anthers placed in carmine-iron-acetic-alcohol become purplish in color, but since the fixative has only a mordanting effect they need to be smeared and stained in the ordinary way. When the chromosomes have been pre-mordanted in this way, they enjoy the fullest advantages of differential staining. The fixative is suitable for all fruit plants except those which do not require much iron, e.g. *Ribes* (see schedule).

Duration of fixation and storage. Very soon after fixation (2–3 hours) satisfactory chromosome staining can be obtained, but the cytoplasm has a granular appearance (Fig. 1). It becomes clear after fixation for 12–24 hours (Figs. 3–5). If the material is stored either in the fixative or in 70% alcohol for a considerable time (1–2 months) it is again difficult to obtain a preparation in which the cytoplasm is clear (Fig. 2). Long storage, however, produces a sharp differentiation between spindle and cytoplasm, a fact which is sometimes useful for studying abnormal spindle development or the positions of the chromosomes relative to the spindle.[1]

To obtain a well-stained flattened cell it is essential to burst the pollen mother-cell wall without actually damaging the cell itself. In preparations made soon after fixation the cells are too delicate and are easily damaged, but on the other hand long fixation makes the cells too hard and it is not possible to burst the wall during the process of heating. The best time must be found by experiment.

[1]Darlington, C. D. and Thomas, P. T. 1937. The breakdown of cell division in a *Festuca-Lolium* deritative. Ann. Botany (London), n. s. **1**, 747–61.

For most fruit plants, fixation for 12–24 hours gives the best results, but for *Ribes*, the cells are so delicate that they cannot be satisfactorily smeared for three days. Hardening can, however, be accelerated by transferring the material to Carnoy.

Strength of aceto-carmine solution and amount of iron introduced. La Cour[2] (1937) advocates dilution of the gentian violet stain for sharp and rapid differentiation of large-chromosome nuclei which take up the stain too readily, and it proves that by far the best root tip preparations of fruit plants also are obtained after dilution of the stain. Hitherto Belling's saturated solution of carmine in 45% acetic acid has been generally advocated in staining schedules. But in the case of aceto-carmine, as in that of gentian violet, a diluted solution makes for easier differentiation in the pollen mother-cells of fruit plants. Generally a one-third strength solution (diluted with 45% acetic acid) is suitable, but the best concentration varies for different plants. Altho iron has been added to the pre-fixative, it is still necessary to add considerably more than usual at the time of smearing. The amount needed again differs markedly for different plants. Pears and apples, for example, require a weak carmine solution and a large amount of iron; *Ribes* requires a weak carmine solution with only a trace of iron. The strength of the aceto-carmine and the amount of iron required are also dependent on the time of storage, weaker amounts being necessary after long storage.

The iron should be introduced during the process of smearing by teasing with iron needles or small scalpels, since aceto-carmine to which much iron has previously been added soon precipitates. The cytoplasm is less liable to become stained, however, if the time between smearing and heating is short; and with plants which demand a considerable amount of iron, it is necessary to dip the needle once or twice into a solution of iron acetate.

It is important to realize that one species or even variety may differ markedly from another in its reaction to aceto-carmine. Indeed, varietal differences may override any characteristic of the species and the importance of adapting the technic to the needs of the individual variety or clone must never be overlooked. In this respect the differences between diploids and triploids of the same species are very remarkable. My colleague, Mr. Raptopoulos, finds it impossible to obtain satisfactory staining for certain triploid cherries unless the acetic-alcohol pre-fixative described above is used, while for diploids and tetraploids the precaution is scarcely necessary.

[2]La Cour, L. 1937. Improvements in plant cytological technique. Botan. Rev., 5, 241–58.

Explanation of figures

All the photographs (except Fig. 4) were made from temporary preparations at a magnification of × 1000 and enlarged to × 3000 for reproduction. Except for Fig. 4 all the material was fixed in the mordant acetic-alcohol fixative described in this paper.

Fig. 1. *Prunus divaricata Moseri* (2n = 16). Bivalents and univalents. Stained in ½ strength carmine 5 hr. after fixation. Cytoplasm granular.

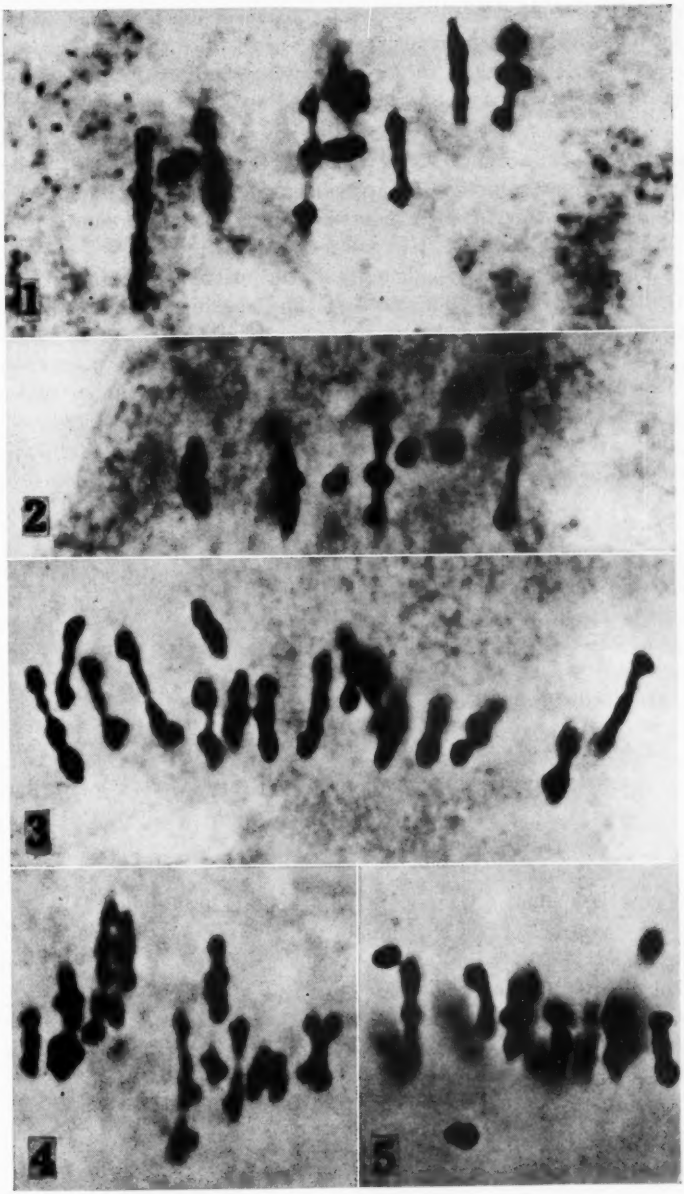
Fig. 2. *Prunus divaricata Moseri.* Bivalents and univalents. Stained in ¼ strength carmine 3 weeks after fixation. Cytoplasm not well differentiated.

Fig. 3. *Prunus spinosa* (2n = 32). 16 bivalents. Stained in ⅓ strength carmine 36 hr. after fixation.

Fig. 4. Duke cherry (2n = 32). Quadrivalents and bivalents. Stained in ⅓ strength carmine 24 hr. after fixation.

Fig. 5. *Pyro-cydonia* hybrid (2n = 34). Bivalents and univalents. Stained in ½ strength carmine 24 hr. after fixation.

Thanks are due to Dr. D. Eissler for the preparation from which Fig. 1 was made.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Critical studies should always be made on the temporary aceto-carmine slides, but permanent slides are useful for record purposes. All the photographs in the accompanying plate except Fig. 4 were made from temporary slides.

Experimental schedule. The following general schedule of aceto-carmine technic is suggested for small chromosomes:

(1) Fix the material in 1:3 acetic-alcohol prefixative and determine the optimum time after fixation when the P.M.C. wall can be burst without damaging the cell.

(2) By diluting Belling's saturated solution of carmine in 45% acetic acid (with 45% acetic acid) find the highest concentration which allows of clear cytoplasm.

(3) Determine the amount of iron which the particular plant needs for optimum chromosome staining without causing the cytoplasm to darken.

(4) If the amount of iron required is considerable, pre-fix in the mordant acetic-alcohol mixture described above.

NOTES ON TECHNIC

CHECKING AND ADJUSTING THE ALIGNMENT OF OBJECTIVES IN THE BINOCULAR DISSECTING MICROSCOPE.—This note concerns two methods found to be useful in keeping the binocular lens systems of this type of microscope in fair alignment; the first is for less refined work, the second is for more accurate work.

In the first method, the check is begun by swinging any one of the three sets of objectives (high, low or middle magnification) into position. Next, an index card is placed upon the dissecting stage. Using *one* eye at a time, the exact periphery of *each* field of vision is outlined by punching small holes into the card with a needle. No less than four holes should be made at the terminals of any two diameters which cross at right angles. When *binocular* vision is again used, the two fields as outlined by the two sets of holes in the card, should nearly coincide. If they do not, the binocular system is out of proper alignment, and the following adjustment is suggested: view the card thru the *left* ocular and see that a set of holes in the card exactly coincides with the periphery of that field; next, *without moving the card* (weight it down), remove the objective barrel and barely loosen the tiny screws which keep the *right* objective plate in position. Then, if the screws have been loosened just enough so that the *right* plate may be moved with slight fingering, and if binocular vision is resumed, this *right* plate may be moved so that the *right* field will finally agree with the *left* field, as denoted by the set of holes in the card. This process must be checked repeatedly by opening and closing the *right* eye and moving the objective plate until the two fields agree.

The second method, which is a more accurate one, involves a refined technic in which a cross-hair ocular or ocular eye piece is used. The method of checking the adjustment remains the same as above, or it may be modified by aligning the *point* of intersection in the cross-hair unit with a tiny *point* made on an immovable index card. In explanation, it may be noted that the diameters of the light diaphragms of the left and right ocular lenses might never be exactly the same. Thus the points of coincidence must of necessity be in the central axis of each lens system. This axis ought then to include the *point of intersection* of the cross-hair and the center of the microscopic field, as indicated by the meeting place of two diameters drawn at right angles upon the index card. In this better method the principle is the same as that suggested above. The *left* side of the binocu-

lar system, containing the cross-hair unit is aligned, the ocular cross upon card cross, with exact central agreement. Next, the unit is moved to the *right* ocular position and the screws attending the *right* objective plate are loosened. This *right* plate is moved so that the crosses *again* exactly agree. Finally, the screws are carefully tightened.—CHARLES O. HATHAWAY, College of William and Mary, Williamsburg, Virginia, and FREDERICK F. FERGUSON, College of William and Mary—V. P. I., Norfolk, Virginia.

RESINS FOR SEALING GLYCERIN MOUNTS [WITH A NOTE ON THE USE OF CLARITE (NEVILLITE V)].—Among materials used for sealing glycerin, glycerin-jelly or glychrogel mounts to make permanent microscopic slides, resins comprise one group usually available in the biological laboratory. To use them, rings of diameter equal to that of the circular cover-slips selected are painted on microscope slides with a fine (No. 1–No. 3) camel's hair brush dipped in the resin; use of a turn-table insures regular rings. Just enough of the glycerin medium to occupy the space in the ring when the cover-slip is placed is applied with a pipette and, if necessary, is warmed to facilitate spreading. The correct amount can be determined only by practice and varies a little with various batches of glycerin jelly and glychrogel. If the preparation is warmed, it should be cooled first, otherwise sealed carefully with resin thinned with xylol or toluol. A second coat should be applied the following day.

In resins employed as seals, clearness and whiteness have been considered desiderata by the writers. Bell's cement, a proprietary seal, fulfills these requirements, but is expensive. Recently a great number of water-white resins have been synthesized of which a few have been tested by biologists as mounting media in place of Canada balsam and gum damar. Three of these are: Clarite (formerly called Nevillite V), developed by the Neville Co. of Pittsburgh, Penna.; Teglac 161, produced by Bakelite, Ltd. of London; and Distrene 80, by Honeywill and Stein, Ltd. of London. The writers have used Clarite (Nevillite V) both as a mounting medium and as a seal. Where great haste is not important, glycerin mounts are made using a store of previously "ringed" slides, and the seal is made using a solution of 60 parts of Clarite to 40 parts of toluol by weight. Where mounts need to be used within a few hours of preparation, the authors use "Cellobalm" made by dissolving 100 g. of ethyl cellulose in one liter of toluol with shaking at intervals over a day or two and then adding 200 g. of Canada balsam. The solution requires no filtration. A more viscous solution can be made by using smaller proportions of toluol.

With Clarite as a mounting medium some workers in our laboratory have found that the toluol solutions recommended by Groat[1] develop bubbles by contraction greater at one side than at others. It is noted, however, that this takes place when fresh slides are warmed for rapid drying. Where slides have dried at room temperature this difficulty is generally obviated. Kirkpatrick and Lendrum[2] in using the water-white Distrene 80 have added a plasticizer, tricresyl phosphate (7.5 cc. tricresyl phosphate, 40 cc. xylol, 10 g. Distrene 80) to prevent retraction and such a modification might be used with Clarite. The superiority of Clarite over Canada balsam and gum damar is most apparent in thick mounts such as whole mounts of large flukes (*Fasciola*), and *Amphioxus* where cells may reach 3 mm. in thickness.—J. L. Mohr and Wm. Wehrle,[3] University of California, Berkeley, Cal.

A New Embedding Schedule for Insect Cytology.—Attention has recently been drawn to the fact that the carbolic acid and water technic simplifies the arduous task of sectioning yolky eggs. These difficulties have been experienced in a study of meiosis in the eggs of various sawfly species, and were successfully overcome by the use of *n*-butyl alcohol and phenol followed by the water treatment. Since much work is now being done on insect cytology and embryology, the time appears opportune to make the schedule available to others.

Petrunkevitch[4] first pointed out that the inclusion of phenol in a fixative gives "a peculiar elastic texture to the tissues, unlike anything produced by commonly used fixing fluids." Slifer and King,[5] however, found that sectioning after the use of one of Petrunkevitch's fixatives provides smooth, clean sections but that "Cytoplasmic details were badly distorted and the chromosomes were almost unrecognizable." By a process of elimination they found that the use of a standard cytological fixative followed by 24 hours soaking in a 4% solution of phenol in 80% ethyl alcohol resulted in equally fine sectioning qualities and at the same time preserved the normal microscopical features. The process of dehydration and embedding was accomplished by means of ethyl alcohol, carbol-xylol and paraffin. After the paraffin had set, the material was blocked

[1]Groat, R. A. Two new mounting media superior to Canada balsam and gum damar. Anat. Rec., **74**, 1. 1939.

[2]Kirkpatrick, J., and Lendrum, A. C. A mounting medium for microscopical preparations giving good preservation of colour. J. Path. & Bact., **49**, 592. 1939.

[3]Employed by the U. S. Works Progress Administration. Aid from this Administration (Official Project 65–108–113, Unit C–1) is hereby acknowledged.

[4]Petrunkevitch, A. 1933. New fixing fluids for general purposes. Science, **77**, 117–118.

[5]Slifer, E. H., and King, R. L. 1933. Grasshopper eggs and the paraffin method, Science, **78**, 366.

and the paraffin pared down until the end of the material was exposed and the whole was then soaked in water for 24 or more hours. Both the phenol and the water treatments are essential; the latter assists the paraffin in gliding over the microtome knife.

In certain species of sawfly the eggs are laid in pine and spruce needles which themselves are extremely tough and consequently add to the difficulty of sectioning the eggs. To prevent hardening during dehydration the *n*-butyl alcohol method of Zirkle was used but only with limited success. Distinctly better sections were obtained by means of the Slifer and King technic, but since the higher concentrations of ethyl alcohol and particularly the xylol have an inherent hardening effect, the following composite schedule was evolved: the results justify its recommendations.

Step	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*
Water	95	90	80	65	50	30	15	5	0	0	0
Ethyl alcohol	5	10	20	35	40	50	50	40	25	0	0
N-butyl alcohol	0	0	0	0	10	20	35	55	75	100	100
Phenol	0	0	0	0	0	0	4	0	0	4	4
Hours	½	½	½	1	1	1	24	1	1	1	—

After step 10 the material and butyl-phenol was placed on an equal amount of solid paraffin, transferred to the oven and changed to pure paraffin after 16 hours or so. Infiltration should be completed in a further four hours. The embedded material was trimmed and soaked as recommended by Slifer and King. Adhesion of the sections was improved by using slides thinly smeared with albumen. The albumen film should be dried and the ribbon floated on with the aid of 35% ethyl alcohol.

The step at which dehydration is commenced varies with the fixative used, e.g., after 2BD, step 1; after Kahle's fluid, step 4; and after Kahle's without water, step 6. Fixation in Kahle's without water, which gives an excellent stain with Feulgen, requires only two hours, so that the whole fixing and embedding process can be completed in approximately two days.—Stanley G. Smith, Department of Genetics, McGill University, Montreal.

Dioxan for Bleaching.—To bleach material fixed in osmic mixtures, either with or without acetic acid, immerse it in dioxan. Bulk material may require up to three days; sections a much shorter time. No additional treatment is required.—J. J. Asana, Gujarat College, Ahmedabad, India.

LABORATORY HINTS
FROM THE LITERATURE

A DEPARTMENT DEVOTED TO ABSTRACTS OF BOOKS AND PAPERS FROM OTHER JOURNALS DEALING WITH STAINS AND MICROSCOPIC TECHNIC IN GENERAL

MICROSCOPE AND OTHER APPARATUS

CROOKS, D. M. **The use of a translongitome in making and interpreting alternative transverse and longitudinal serial sections.** *Science*, **91**, 150. 1940.

Translongitome is the name given to a new microtome attachment developed for the purpose of obtaining transverse and longitudinal sections from the same block. Such a two-plane cutting attachment can be used in a rotary or a sliding microtome. The sections come off in the form of a continuous ribbon in which they have been cut alternately crosswise and longitudinally. The device fastens into the microtome clamp, and consists fundamentally of a hinged sector capable of swinging thru an angle of 90° in a plane parallel to the knife edge. It is, of course, necessary to set the microtome to cut one half the thickness desired as each face of the block is cut on alternate strokes. The translongitome is manufactured under patent rights, and priced at $22.50.—*J. A. de Tomasi.*

ECKEL, EARL E. **Cover slip dispenser.** *J. Lab. & Clin. Med.*, **25**, 882–3. 1940.

A machine is described which ejects cover slips one at a time in a position to be grasped by the edges without smearing the surfaces. The machine is composed of a plunger of correct thickness running on a horizontal plane thru a box containing cover slips, the edges of the box being raised sufficiently in the direction of the motion of the plunger to allow only one cover to be pushed out of the box. The box can be bent into shape from scraps of stainless steel or monel metal. The slit is adjustable for thickness of cover slip. The box and runway for the plunger is soldered to a heavy metal bar. The plunger is cut from the 0.006-inch blade of a thickness gauge. A pin is soldered to each end of the plunger and one to the back of the base to permit a rubber band to draw back the plunger.—*John T. Myers.*

JOHN, K. **Ein neues Universalgerät.** *Zts. wiss. Mikr.*, **56**, 371. 1939.

A new universal microscope manufactured by Fuess of Berlin is described. It differs from the well known Leitz "Panphot" in placing the photographic portion of the apparatus to the left of the microscope on the same plane instead of superimposing the camera over the latter. An ingenious arrangement permits the light to be diverted either to the left for photomicrography or to the right for microprojection upon the drawing board. The illuminating unit and accessories are permanently built in and are suitable for opaque illumination as well as for work with the polariscope.—*J. M. Thuringer.*

MOSEBACH, GEORG. **A microprocess for the cryoscopic investigation of succulent tissues.** *Ber. deut. botan. Ges.*, **58**, 29–40. 1940.

A microcryoscope is described by means of which osmotic concentrations may be determined on pieces of succulent tissue having a volume smaller than 1 cu. mm.—*Merritt N. Pope.*

PHOTOMICROGRAPHY

ARMITAGE, F. D. **An eyepiece camera for miniature negatives.** *The Microscope*, **4**, 124–7. 1940.

The camera consists of two tubes and a focusing screen. The lower tube fits over a fiber washer that is placed over the eyepiece end of the body tube or draw-

tube, forming a light-tight joint. Between the two tubes of the camera are two diaphragms of fixed aperture; between these slides a metal plate serving as a shutter. At the top of the camera is a brass-bound wooden holder for the focusing screen or the double-sided plate-holder. The focusing screen is ruled in squares of ½ cm. sides; in its center is a cover-glass cemented with Canada Balsam so that a focusing lens can be used. The negatives (4.5 x 6 cm.) will bear considerable enlargement.—*C. E. Allen.*

AUER, A. **Die Verwendung des Rollfilms und die Messung der Belichtungszeit in der Mikrophotographie.** *Zts. wiss. Mikr.*, **56**, 259. 1939.

To determine exposure time for photomicrography the use of a small extinction type exposure meter such as the Zeiss Icon "Diaphot" is recommended. The aperture of this disc-shaped exposure meter is brought directly over the eye-piece. The gray wedge is rotated until the desirable microscopic detail just disappears. An exposure table is then made based upon known correct exposures and the corresponding meter reading for every lens combination used. For cameras with bellows, additional calculations must be made for various extensions.—*J. M. Thuringer.*

MICROTECHNIC IN GENERAL

BEYER, E. M. **Trichrome stain for astrocytes.** *Amer. J. Clin. Path., Tech. Suppl.*, **4**, 65–8. 1940.

A trichrome stain for astrocytes is described which gives results as good as those obtained with gold or silver impregnation and is suitable for bulk staining. It is better than Masson's trichrome stains for color photomicrography using Kodachrome or color separation. The method is as follows: Fix in formalin. Double embed (Beyer: Amer. J. Clin. Path., Tech. Suppl., 2, 173, 1938). Cut sections 5 μ. Remove paraffin. Run thru alcohols to water. Apply Muller's mordant, 3–12 hr. Wash in 3 changes of tap water and 1 of dist. water. Apply Hansen's hematoxylin, 5 min. or longer (Soln. A: ferric alum, amethyst cryst., 10 g.; ammonium sulfate, 1.4 g.; dist. water, 150 cc., heated below boiling until dissolved, and cooled. Soln. B: hematoxylin, 1.6 g.; dist. water, 75 cc., dissolved by heat, and cooled. A is added to B gradually with constant stirring, and when deep violet, as seen on filter paper, is heated slowly to boiling and boiled 30 sec., then cooled in ice water.) Wash in running tap water, 2 min. Differentiate in 2% acid alcohol, 15 sec. Wash in tap water. Rinse in ammonia water (1–2 drops to 100 cc.). Wash in dist. water. Stain in ponceau-fuchsin, 5 min. (Soln. A: ponceau de xylidine, either Eimer and Amend, Krall or Grübler, 1 g.; glacial acetic acid, 1 cc.; dist. water, 100 cc. Soln. B: acid fuchsin, Commission cert., 1 g.; glacial acetic acid, 1 cc.; dist. water, 100 cc. To 90 cc. of 0.2% acetic acid 6.6 cc. of soln. A and 3.3 cc. of soln. B are added). Wash in 2 changes of tap water and 1 of dist. water. Stain 5 min. in: phosphotungstic acid, 3 g.; orange G (Hollborn), 2 g.; dist. water, 100 cc. Mix in a mortar. Rinse quickly in 1 change of dist. water. Put in 0.5% Wasserblau (Grübler, sold by Pfaltz and Bauer) 10 to 15 min. Rinse in dist. water containing 1% of the Wasserblau staining solution. Run thru 2 changes of 95% alcohol, 2 changes of abs. alcohol, and then xylol. Mount in neutral balsam or gum damar.—*G. H. Chapman.*

CIARDI-DUPRÉ, G. **Accorgimenti per allestire economicamente serie istologiche.** *Monit. Zool. Ital.*, **50**, 314–5. 1940.

An inexpensive substitute for slides and cover slips to mount large sections of embryonal and fetal tissue is described. Sections are mounted on old photographic glass which has been cut to the proper size and from which the gelatin has been peeled. Sheet cellophane, weighing 140–160 g. per sheet measuring 1×1.50 m. is used to cover the sections. Damar balsam is recommended as a mounting medium, and the cellophane, cut to appropriate size, is applied as one would a cover slip. It is important that the cellophane be under pressure for at least 24 hr. so as to obtain a level surface. The author suggests pressing between two thick pieces of cardboard held by a pair of elastic bands.—*A. B. Dawson.*

KARR, J. W. **A substitute for balsam and damar.** *Amer. J. Clin. Path., Tech. Suppl.*, **4**, 70. 1940.

Nevillite (Neville Company, Neville Island, Pittsburgh, Pa.) is suggested as a mounting medium.—*George H. Chapman.*

MC NAMARA, W. L., MURPHY, BERTA, and GORE, W. A. **Method of simultaneous fixation and decalcification of bone.** *J. Lab. & Clin. Med.*, **25**, 874–5. 1940.

The following method of simultaneous fixation and decalcification can be used for the majority of special staining procedures: Cut sections of bone not more than 8–10 mm. thick with a scroll saw. Place them in the decalcifying solution at 37° C. Dissolve 10 g. of $HgCl_2$ in 300 cc. of dist. water with heat and cool it. Dissolve 30 g. of trichloracetic acid in 100 cc. of water and add 5 cc. HNO_3, 40 cc. formalin and 50 cc. 95% alcohol. (Mix the two solutions.) Change this solution daily until tissue is soft. Keep in running water for 24 hr. (or in 2% NH_4OH for 12 hr. and running water for 24 hr.) Place in 80% alcohol, 2 hr.; 95% alcohol, 6 hr.; abs. alcohol, 16–24 hr.; xylol, 5–10 min.; cedar oil, 24 hr.; xylol (2 changes), 30 min.; 56°–58° paraffin (3 changes), 4–6 hr.; and embed. The average bone decalcifies in 3–5 days. If longer than 7 days is required, nuclear staining is impaired. By this technic tissue distortion is minimal and the staining capacity of the nuclei is undiminished.—*John T. Myers.*

O'BRIEN, H. C., and HANCE, R. T. **A plastic coverglass, isobutyl methacrylate.** *Science*, **91**, 412. 1940.

Natural resins, like Canada balsam and gum damar, harden slowly and turn yellow in time. Nevillite, or Clarite, recently suggested by the General Biological Co., dries rapidly and is practically colorless. Isobutyl methacrylate, a plastic made by the du Pont Co. and costing about $1.00 a lb., is similarly found to dissolve in benzene and xylene, giving a colorless solution. Moreover, it dries very hard within 5–10 min. and yields a preparation somewhat more brilliant than those mounted in Clarite. Its refraction index is 1.477, very near that of glass. Isobutyl methacrylate may also be used to replace the coverglass by simply dipping the slide into a thin solution of the plastic and draining off the excess. Immersion oil does not affect its surface, which can be wiped clean with alcohol and soft paper. The film produced is very thin, thus interference with light transmission is very small; but it will scratch. Scratches are removed by dipping the slide into the same plastic solution. Lastly, the film obtained can be written on with ordinary ink for purposes of labeling.—*J. A. de Tomasi.*

WARD, MARGARET C. **Gum damar in place of thin celloidin for frozen sections.** *Amer. J. Clin. Path., Tech. Suppl.*, **4**, 71. 1940.

Gum damar, used to affix frozen sections to slides, does not interfere with staining. The method described is as follows: Coat the slide with gum damar. Float section onto the slide and drain. Flush with 70% alcohol and blot with a blotter moistened with 70% alcohol. Stain as usual. Keep the slide horizontal while in xylol because it tends to loosen the section.—*G. H. Chapman.*

DYES AND THEIR BIOLOGICAL USES

FIGGE, F. H. J. **Squid melanin: A naturally occurring reversibly oxidizable pigment.** *Proc. Soc. Exp. Biol. & Med.*, **44**, 293–4. 1940.

The ink of the squid (*Loligo pealii*) is a highly concentrated suspension of melanin, the particles of which are visible only by dark field illumination. Comparisons between this melanin and synthetic dopa melanin indicate marked reduction with sodium hydrosulphite and re-oxidation with potassium ferricyanide in both instances, whereas natural melanin exhibits more sluggish reactions. Squid melanin contains some substance absorbing about 10% of the light which is not reversibly oxidizable.—*M. S. Marshall.*

ORR, J. W. **The histology of the rat's liver during the course of carcinogenesis by butter-yellow (*p*-dimethylaminoazobenzene).** *J. Path. & Bact.*, **50**, 393–408. 1940.

The dye "butter-yellow", obtained from British Drug Houses, Ltd. under the name "dimethyl yellow", was dissolved in oil and then added to the diet for the induction of visceral neoplasms.—*S. H. Hutner.*

ANIMAL MICROTECHNIC

BRUNSCHWIG, A., SCHMITZ, R. L., and JENNINGS, S. **Selective localization of Evans blue (T1824) in subplacental portions of entoderm in the rat.** *Proc. Soc. Exp. Biol. & Med.*, **44**, 64–6. 1940.

The injection of pregnant white rats with 4 mg. of trypan blue was followed by localization as small blue granules in the columnar cells of the subplacental portions of the visceral entoderm. The experimental check followed observations of the localization of Evans blue (T1824), which accumulated in the macrophages and fibroblasts of the stroma around malignant but not benign tumors, following the injection accidentally of a pregnant rat.—*M. S. Marshall.*

DIGGS, L. W. and PETTIT, V. D. **A comparison of methods used in the detection of the sickle-cell trait.** *J. Lab. & Clin. Med.*, **25**, 1106–11. 1940.

Five methods of detecting sickle cells were compared. The best one is the following moist stasis technic of Scriver and Waugh (Canad. Med. A. J., **23**, 375, 1930): Place a rubber band around the proximal third of the finger and allow it to remain for 5 min. Puncture the distal end of the finger, seal a drop of the dark blood under a cover slip with vaseline and examine microscopically.—*John T. Myers.*

GOMORI, G. **A method for staining of carious lesions in teeth.** *Proc. Soc. Exp. Biol. & Med.*, **44**, 250–3. 1940.

The following method permits rapid and accurate work, with human teeth or with large numbers of rat jaws: Fix in 80–95% alcohol or neutral formalin and wash in repeated changes of dist. water. Impregnate with 0.25–0.5% $AgNO_3$ for 12–24 hr. and wash for at least 24 hr. Reduce in 5% $Na_2H_2PO_2$ for 24 hr. and wash several hours in tap water. Fix in 2% $Na_2S_2O_3$ ("hypo") for 12 hr. and wash under the tap several hours. Stained jaws may be dehydrated in alcohol, cleared in cedar oil and examined directly under the dissecting microscope or they may be decalcified in 5–10% sulfosalicylic acid (not mineral acids) and embedded in celloidin. Carious areas are black; healthy areas are unstained.—*M. S. Marshall.*

IGNESTI, UGO. **Avvertenze per la colorazione con la fucsina di Ziehl di materiale fissato in liquidi contenenti acidi.** *Monit. Zool. Ital.*, **51**, 80–2. 1940.

A modification of the Vannucci-Mallory method is proposed in which the acid fuchsin is replaced by Ziehl's fuchsin according to the method of Gallego. This modification permits differential staining following fixation with alcohol or fluids containing acid (Susa, Gilson-Carazzi, Zenker acetic, picro-sublimate, Sanfelice, and Bouin).

The essential modification is the omission of acetic acid from the solutions used in steps 1 and 3 of the following procedure: 1) Stain for 3–5 min. in dil. carbol fuchsin (5 g. Ziehl's fuchsin, dissolved in 10 cc. dist. water). 2) Wash in dist. water. 3) Differentiate for 5 min. in dil. formalin (formalin, 3 g., dissolved in dist. water, 10 cc.). For the rest of the steps, the original method is followed.

If nuclear staining is too intense, the solution in step 1 may be made with 2 g. Ziehl's fuchsin, and the time of staining reduced to 1–3 min.—*A. B. Dawson.*

JÄGER, R., and JÄGER, F. **Fluoreszenmikroskopie im auffallenden Licht unter besonderer Berücksichtigung der Struktur der Oberfläche der lebenden Haut und der Vereinfachung der Hilfsmittel.** *Zts. wiss. Mikr.*, **56**, 273. 1939.

This is a technical description of various methods employed in obtaining sharp surface contour pictures of the epidermis. The latter is rendered sensitive to ultra-violet light by washing the parts to be examined with a dil. solution of some flurochrome such as Primulin "O" (Grübler) or Auramine (Hollborn). The usual opaque illuminators are used.

The method should find various practical applications in studying the effects of soaps and cosmetics, industial hazards, etc., upon the epidermis.—*J. M. Thuringer.*

JUGE, JEAN. Les potentialities morphogenetiques des segments du membre dans la regeneration du Triton (Autopode). *Rev. Suisse de Zool.*, **47**, 65–133. 1940.

A method for contrast staining of bone and cartilage in cleared specimens is described as follows: Fix 24–48 hr. in 4% formalin. Wash in running water several hours. Transfer to 70% alcohol for 24 hr. Remove the skin. Stain 1–3 hr. in acetic methyl green (methyl green, 3 g.; 70% alcohol, 100 cc.; acetic acid, 2 drops). Differentiate in several changes of 70% alcohol several hours until the desired color is reached; 95% alcohol, 2 hr.; abs. alcohol, 2 hr. Stain 12–24 hr. in alizarin (saturated solution of alizarin in abs. alcohol, 1 cc.; abs. alcohol, 100 cc.; acetic acid, 5 drops). Transfer to abs. alcohol for 2 hr. Clear in toluol or benzol for ½ hr.

Material may be preserved in a mixture of 27 cc. methyl salicylate with 7 cc. isosaphrol, or in methyl salicylate alone. Cartilage appears green (but loses its color at the end of several months); bone is red.—*A. B. Dawson.*

KAUFMANN, W. Occurrence of special cell groups at vascular pole of glomerulus in mammalian kidneys. *Proc. Soc. Exp. Biol. & Med.*, **44**, 227–30. 1940.

This is a report of a study of the juxtaglomerular corpuscles of Goormaghtigh, composed of agglomerations of cells. The author recommends fixation in Bouin's or Zenker's solution while fresh, paraffin embedding and sectioning serially, 4–6 μ. Hematoxylin-eosin fails to give adequate detail, Masson's trichrome stain is better and Mallory's phosphotungstic-acid-hematoxylin reveals good nuclear detail. Cytoplasmic vacuolization is visible by this method or with Mallory's anilin blue connective tissue stain. Intercellular fibrils are well brought out with Masson's stain or better with silver stains.—*M. S. Marshall.*

LANDAU, E. Quelques réflexions sur les procédés d'imprégnation des neurofibrilles par le nitrate d'argent. *Bull. d'Histol. Appl.*, **17**, 65–80. 1940.

A method is described for the silver impregnation of paraffin sections from the nerve fibers of the central nervous system, as follows: Use material that has been fixed in formalin. Fasten the sections to slides with a very weak solution of gelatin, heating to an optimum temperature of 35° C. Remove paraffin and place in a 10% solution of neutral formalin 12–24 hr. or longer, protected from light. (The authors do not consider treatment with 95% alcohol desirable before impregnation.) After removal from formalin, rinse in dist. water, and transfer, in the dark, to a 20% solution of $AgNO_3$. Hold in an oven at 35–40° C. for 1–2 hr. according to the thickness of the section. Rinse 1 sec. in dist. water and transfer to 20% ammoniacal silver. After 5 min. rinse briefly and reduce the silver by several drops of 1% neutral formalin. Repeat at intervals, controlling the reduction of the silver under the microsope.

The impregnation is continued until the desired result is obtained. The reduction may then be stopped by formalin, by a thoro washing in dist. water, or by the method proposed by Gros. After toning in $AuCl_3$ and fixing in $Na_2S_2O_3$, the sections may be differentiated in a ½% aq. solution of potassium ferricyanide or 10–15% KI. When the differentiation is finished, the section is washed several hours in running water, dehydrated, and mounted either in Canada balsam or in the following fluid preferred by the authors: dist. water, 30 cc.; gum arabic, 30.0 cc.; refined sugar, 30 g.; glucose, 5 g.

This method has been used successfully for the cerebral cortex, the cerebellum and the bulb.—*Jean E. Conn.*

MC CARTER, JOHN C. A silver carbonate method for oligodendrocytes and microglia for routine use. *Amer. J. Path.*, **16**, 233–5. 1940.

The following silver method is recommended for tissue of the central nervous system: Fix in 10% formalin. Treat frozen sections (20–25 μ) with a 1% solution of strong ammonia in dist. water for a few minutes, if the material is recently fixed, or over night if fixed for several weeks. Without washing, transfer to 4% aq. HBr (10% soln. of conc. hydrobromic acid) at 37° C. for 1 hr., wash in 2 changes dist. water and put into 5% aq. Na_2CO_3. Add an equal volume of 5%

aq. ammonium alum (disregard ppt.) and leave the sections in this mixture for 1 hr. to 2–3 days. Wash in 2 changes dist. water and stain in Hortega's strong silver carbonate solution (5 cc. 10% $AgNO_3$, plus 20 cc. 5% Na_2CO_3, plus strong ammonia water to just dissolve ppt., filter and add 20 cc. dist. water) for 2–5 min. Reduce, without rinsing, in 1% formalin and agitate by blowing on the sections. Wash well in dist. water, gold-tone and fix in hypo in the usual manner. Dehydrate the sections after putting on the slide and cover in balsam. The method combines modifications of Hortega's method for oligodendrocytes and microglia with those of Globus and Penfield and adds the ammonium alum mordant; the time the sections remain in the first ammonia bath is varied to suit the time of fixation.—*H. A. Davenport.*

REXED, B., and WOHLFART, G. **Über Färbung mit gepufferten Säurefuchsinlösung.** *Zts. wiss. Mikr.*, **56**, 212–5. 1939.

The authors attribute the extreme variability of the action of acid fuchsin as used in Mallory's connective tissue stain to the varying pH of its solution. The reaction of a freshly prepared 0.1% solution of acid fuchsin is pH 4.49, and any alkalinity raises the pH sufficiently to render the stain inert. The following recommended formula gives a solution of pH 3.29±0.01: acid fuchsin, 1.0 g.; N/10 HCl, 60.0 ml.; dist. water, 900 ml.; Sörensen's citrate solution, 40 ml. (citric acid crystals 21.0 g., N/1 NaOH 200 ml., dist. water to make 1 L.).

Practically all organs stain in range of pH 3–4. The red blood cells alone stain at pH 5–7.—*J. M. Thuringer.*

RÖHLINGER, H., and REITZ, M. **Die Methode der Schnittveraschung und ihre Bedeutung für die physikalisch-therapeutische Forschung.** *Zts. wiss. Mikr.*, **56**, 361. 1939.

The importance of the spodogram in dermatology for the control of physical therapy measures is discussed. Every functional change of the skin is contingent upon a shifting of its electrolytic components.

The presence of calcium, magnesium, phosphates, iron, and silicic acid are determined as follows: Fix the living tissue in abs. alcohol 48–72 hr. and imbed in paraffin. Cut sections 10 μ, mount with dist. water and incinerate with aid of microincinerator. Postmortem material is unsuitable because of its altered cell permeability, diffusion phenomena, and change in pH. The preparations are unstable and photomicrographs must be made immediately.

To determine total Ca salts: breathe gently on the spodogram to convert the water soluble Ca salts into carbonates which are practically insoluble. The residue may be washed from the preparation with dist. water.

The Mg pictures obtained after previous removal of Ca salts are less definite than when made from the fresh spodogram. It is demonstrated by treating the spodogram for 3 min. with a 1% tetraoxyanthrachinon solution and removing the remainder with a 10% solution of antipyrin. Lastly, the sections are treated for 30–40 min. with dist. water (40–50° C.) using several changes.

The demonstration of phosphates is more difficult because of precipitate formation on the spodogram; however, they may be shown in the total ash as well as Ca picture. To overcome this difficulty a drop of abs. alcohol may be placed carefully on the spodogram and cover slip. The reagent ferric acetate (concentration not mentioned) is dropped at the side of the cover slip and drawn thru by holding a bit of filter paper to the opposite side. A few drops of alcohol mixed with the reagents hastens this process. This procedure should be repeated 3 or 4 times. A solution of 1% acetic acid in 33% alcohol is similarly drawn thru. Ferric phosphates are formed while other salts are removed by acetic acid. The iron salts are rendered visible by drawing thru first 2–3 drops of a potassium ferrocyanide solution followed by 4–5 drops of 1% HCl.

Iron may be demonstrated with the Berlin blue or still better the Turnbull reaction, as follows: precipitate with ammonium sulphide, add 20% potassium ferrocyanide in the presence of HCl and observe the blue color reaction.

Silicic acid is isolated by treating the spodogram ½–1 min. with 1–2% aq. HCl. The remainder of the spodogram consists of silicic acid since all other salts are dissolved and washed away by HCl.—*J. M. Thuringer.*

ROSKIN, G., and KIRPICHNIKOVA, E. **Coloration vitale par ionophorèse.** *Bull. d'Histol. Appl.*, **17**, 146–7. 1940.

A method is described for vital staining of animal cells by means of ionization. Colored ions are introduced into the living organism by means of a continuous current from a storage battery having an intensity of 0.25 milliamperes for an electrode of 1 sq. cm. Tin plates are used for electrodes with the active one a little smaller than the other. Various dyes can be used; e.g., methylene blue which has a colored positive ion. The electrodes are attached to the animal after the hair has been removed. A pad of gauze soaked in a 0.5–1.0% solution of methylene blue is placed under the active electrode, and gauze wet with water is placed under the other electrode. The current is allowed to pass for 20–30 min.

Coloration can be observed after the first electrification, but it becomes more intense after this has been repeated on several successive days. With white mice coloring has been observed, not only in the cells of the skin, but also in the outer layers of the muscles. After killing the animal, the tissues are fixed in the following solution: Sat. aq. picric acid, 6 parts; sat. aq. ammonium molybdate, 2 parts; formalin, 2 parts. They are then passed thru alcohol and embedded in paraffin.

This method can also be used for vital staining of nerves.—*Jean E. Conn.*

SCHORR, E. **A new technic for staining vaginal smears.** *Science*, **91**, 321. 1940.

The conventional hematoxylin-eosin-water-blue technic of Papanicolaou for vaginal smears is not entirely satisfactory when stress is put on the detection of cornification, a cytoplasmic change taken as a measure of ovarian function. The following modification of the Masson trichrome stain has, however, proved useful, because the sequence of contrasting colors produced is comparable to a chemical titration of the cell charge of the smear. Fix (no time given) the wet smear in 95% alcohol-ether, 1:1; run down to water. Stain 2 min. in Harris' hematoxylin; rinse 4–5 times in water, and let stand 5 min. in running water. Stain 5 min. in ponceau-acid-fuchsin-orange-G; rinse 3–4 times in water. Mordant 10 min. in 3% phosphotungstic acid; rinse 3–4 times in water. Counterstain 8 min. in light green; do not wash. Acidify 3 min. in 0.25% acetic acid; do not wash. Dehydrate, clear, and mount. For the composition of the trichrome stain reference is made to Foot's description (Amer. J. Path., **14**, 245, 1938).—*J. A. de Tomasi.*

TAFT, A. E. **A supplementary method for the study of Arachnopia.** *Science*, **91**, 272. 1940.

The usual preparations of brain and cord tissue give an inadequate idea of structure and histological relations of leptomeninges. Such stained preparations can, however, be supplemented with others which, prepared by the following simple technic, will often yield further valuable information: Float fragments of fresh or formalin-fixed tissue from water onto a glass slide, add a drop of glycerin, and put cover in place, with the application of some pressure. The material is thus made suitable for dark field work, a technic especially valuable when the study of vascular arrangement and meningeal concretions is called for.—*J. A. de Tomasi.*

TAKEYA-SIKO. **Markscheidenfärbemethode am Gefrierschnitt.** *Zts. gesam. Neurol. u. Psychiat.*, **169**, 216–9. 1940.

As a supplement to Schroeder's article (abs. Stain Techn., **15**, 37) two methods for myelin sheaths are presented. The first is the Sugamo method as follows: Fix in 10% formalin; wash in running water 12–36 hr. Cut frozen sections (about 30 μ). Place 3–5 min. in 50% alcohol (agitate well); transfer to dist. water, then mordant 6–15 hr. at 37° C. in $K_2Cr_2O_7$, 5 g.; chromalum, 2 g.; dist. water, 100 cc. with sections flat and submerged in the fluid. Rinse 2–3 times in dist. water and stain 2–6 hr. at 37° C. in Kultschitsky's acetic hematoxylin. Transfer to water (use perforated spatula as sections are brittle); wash thoroly; then differentiate in 0.3–1.0% $KMnO_4$ 20–30 sec., one section at a time. Rinse in dist.

water and treat with a fresh mixture of equal parts of 1% oxalic acid and 1% Na_2SO_3. Wash thoroly in tap water; dehydrate; clear in creosote-xylene and mount in balsam. The Yatusiro method is like the first with the following exceptions: Omit the mordant; stain in aged Delafield's alum-hematoxylin 12–24 hr.; differentiate in borax 2 g., potassium ferricyanide 2.5 g., water 100 cc.—*H. A. Davenport.*

WOLF-HEIDEGGER, G. Die Anwendung von Kava-Kava bei der Fixierung des Dünndarms und anderer Hohlorgane. *Zts. wiss. Mikr.*, **56**, 417. 1940.

Postmortem contractions of smooth muscle in the alimentary tract of mammals produce distorted histological pictures. To overcome this difficulty an infusion of powdered Kava-Kava root (*Piper latifolium*) is recommended.

The procedure is as follows: Heat 250 ml. Ringer's solution slowly to 80° C.; add 15 g. Kava-Kava powder, agitating constantly. When cooled to 37° C., take a few ml. of this solution and add a small amount of Merck's diastase, mix thoroly and add to the remainder of the Ringer's. Let digest in incubator for 2½ hr., filter, discard ppt., and proceed to inject animal. Any of the usual fixatives may follow this treatment.

The results were excellent on cats while rabbits receiving the same preliminary treatment did not respond to the Kava-Kava relaxation which may be attributed to their being vegetarians.—*J. M. Thuringer.*

PLANT MICROTECHNIC

GORDON, W. E. A labor-saving technique for leaf samples in histological work. *Science*, **91**, 390. 1940.

For the purpose of recording the source of each leaf sample and maintaining its identity thruout the various manipulations in the laboratory, the following procedure is recommended: By means of a crow quill pen, put duplicate numbers in India ink on fresh leaves in the region from which the sample is to be taken. Punch out a disk-like portion which includes one of these numbers, and use this as a sample to embed and store for future sectioning. The other number is left to record the source and location of the sample taken; the leaf bearing it is pressed and dried for filing. As many as 15 such samples from the same specimen can be preserved, fixed, dehydrated and embedded at the same time with the sample bearing the number at the bottom of the block, so that it is legible thru the thin layer of paraffin. The numbers in ink are not washed off by chrom-acetic, or formol-acetic-alcohol fixatives, nor by the common alcohols, dioxan, or chloral hydrate.—*J. A. de Tomasi.*

HILLARY, B. B. Use of the Feulgen reaction in cytology. I. Effect of fixatives on the reaction. *Botan. Gazette*, **101**, 276–300. 1939.

"In vitro" experiments with nucleic acid impregnated agar blocks using four different types of fixatives showed that the Feulgen nucleal stain gives two types of staining curves, depending on the presence or absence of chromic acid in the fixative. With fixatives not containing chromic acid, the maximum stain is produced by hydrolysis at 60° C. extending from 4 to 8 min.; after that time a gradual falling off of the stain takes place, till at 30 min. no stain is visible. With fixatives containing chromic acid, the maximum stain is produced by hydrolysis at 60° C. for from 5–30 min. That this greater retention of the stain with increased hydrolysis is due to chromic acid was established by testing the individual ingredients of the fixatives separately. Most of the ingredients of the fixatives, if they are present at the concentration normally used, do not interfere with the stain. Tannins, which occur widely in plant cells and which have been reported to inhibit the stain, were found to have no inhibitory effect unless a fixative was used that contained formalin. The nuclei of various plants from different divisions thruout the plant kingdom when treated in the same manner as the agar blocks showed the same type of staining curves. Causes of the previously reported negative stain reactions in many of these plants are discussed.—*B. B. Hillary.*

THOMAS, J. WARRICK. **A comparison of cedar oil and other materials in the making of slides of atmospheric pollen.** *J. Lab. & Clin. Med.*, **25**, 1086–90. 1940.

Comparative studies were made of atmospheric pollen slides prepared with a glycerin jelly, glycerin vaseline, corn oil, almond oil and cedar oil. Glycerin jelly, containing a trace of methyl green (proportion not given) is desirable where structural detail is desired, but not for routine use. Cedar is preferable to other oils because it remains in the slide area to which it is applied, is more adhesive and makes the pollen grains more translucent. Cedar oil preparations may be filed either mounted with Canada balsam and cover slip, or unmounted in dust proof slide boxes.—*John T. Myers.*

MICROÖRGANISMS

HORVATH, J.v. **Die Bedeutung des Zuchtwassers und der Fixierung in der Mikrotechnik der Protozoen. I. Die Wirkung des Eisennitrates bei Anwendung von Anilinfarben.** *Zts. wiss. Mikr.*, **56**, 291. 1939.

The staining of protozoa can be influenced by adding 5 drops of 0.13% $Fe_2(NO_3)_3$ to 10 ml. of the hay infusion or other culture medium in which they are growing. The action of the iron salt is said to be entirely unlike that of a mordant since it has no effect after fixation. It may be used in relatively high concentrations "in vivo" without affecting the viability of the organisms, and may be followed with one of the usual fixing fluids suitable for protozoa. The specifity of the various stains may be altered by regulation of the amount of $Fe_2(NO_3)_3$ added.—*J. M. Thuringer.*

LEVADITI, C., REINIÉ, L., STAMATIN, LE-VAN-SEN, and BEQUIGNON, R. **Ultravirus et fluorescence. Le virus vaccinal.** *Ann. Inst. Pasteur*, **64**, 359–414. 1940.

The following modified Hagemann technic is offered for the use of fluorescent dyes in the microscopy of viruses: Smear thick suspensions of elementary bodies on a slide, dry 1 hr. at 37° C. Agitate slides in dist. water for 10 min., dry 1 hr. at 37° C. Stain 5 min., rinse in dist. water, examine dry preparations by fluorescence. The authors used the Zeiss cardioid dark-field apparatus supplied with quartz stage accessories to enable use of an ultraviolet light source. The best dye, thioflavine, and the less suitable dyes, primuline, thioflavine S, auramine O, rhodamine B, trypaflavine, uranine A and eosin G, were obtained from Hoerlein. The elementary bodies are brilliantly fluorescent against the dark background.

Silver impregnation staining methods afford a good check on fluorescence methods.—*S. H. Hutner.*

HISTOCHEMISTRY

CRÄMER, G. **Ein Verfahren, Nitrate im Gewebe sichtbar zu machen.** *Zentbl. allg. Path.*, **74**, 241–4. 1940.

The reagent, diphenyl-endo-anilo-dihydro-triazole (called "Nitron" by Busch), forms insoluble salts with nitrates and these can be demonstrated in tissue by examination with polarized light. The method is as follows: Dissolve 10 g. "Nitron" with 100 cc. of 5% acetic acid, heat (temp. not given). Cut fresh (unfixed) tissue with a freezing microtome (cool knife) and receive the section on a slide. Put 1–2 drops of hot "Nitron" solution on a cover glass and cover the section with it. Store ½ hr. in a refrigerator to assist crystallization of the nitrate. Examination with polarized light reveals the presence of nitrates as doubly refractive zones. Methylene blue can be added to the "Nitron" reagent if desired. Sections must be examined immediately after removal from the refrigerator. A discussion of interfering substances is given. Further information: Busch, M., Ber. deut. chem. Ges., **38**, 861.—*H. A. Davenport.*

STAINS RECENTLY CERTIFIED

In the table below is given a list of the batches of stains approved since the last one listed in the July number of this Journal.

STAINS CERTIFIED JUNE 1, TO AUG. 31, 1940*

Name of dye	Certification No. of batch	Dye Content	Objects of tests made by Commission†	Date approved
Phloxine	CPh–3	90%	For histological staining	June 17, 1940
Wright stain	CWr–13	....	As blood stain	June 27, 1940
Rose Bengal	CRb–3	80%	As bacteriological stain	June 28, 1940
Brilliant cresyl blue	NV–19	55%	For vital staining of blood	July 8, 1940
Methyl orange	LM–1	91%	As histological counterstain	July 16, 1940
Methyl orange	NM–6	91%	As histological counterstain	July 16, 1940
Pyronin Y	NP–8	52%	As constituent of Pappenheim stain	July 22, 1940
Methylene blue thiocyanate tablets	NAt–2	1.31–1.65%	For use in reduction test of milk	July 23, 1940
Hematoxylin	FH–16	....	As histological and cytological stain	Aug. 2, 1940
Fast green CF	LGf–1	93%	As histological and cytological counterstain	Aug. 10, 1940
Malachite green	NMg–8	93%	As histological and bacteriological stain	Aug. 12, 1940
Giemsa stain	NGe–6	....	As blood stain	Aug. 21, 1940
Tetrachrome stain	NMn–11	....	As blood stain	Aug. 31, 1940

*The name of the company submitting any one of these dyes will be furnished on request.

†It is not to be inferred that these are the only uses for which each of these samples may be employed. The Commission ordinarily tests each dye for such of its common uses as seem to give the most severe check as to its staining value. Certification does not in any instance, however, imply approval for medicinal use.

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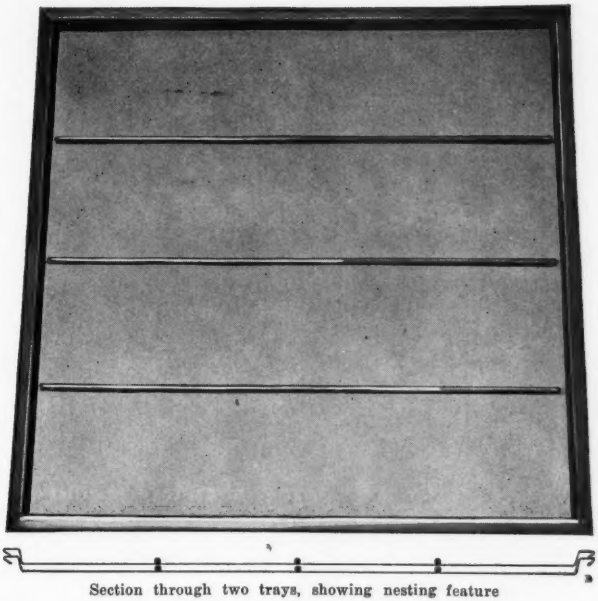

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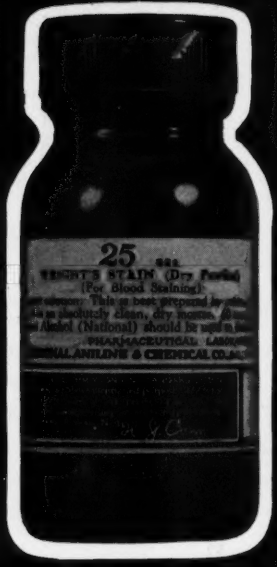

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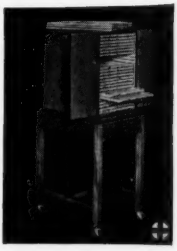

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No. 2, per oz.	**1.65**	**1.65**	**1.65**	**1.65**	**1.65**
Code Word	*Junvx*	*Junsp*	*Juocj*	*Juofd*	*Juoix*

10% discount in carton of 6 oz., one catalogue number, one size and thickness only
15% discount in lots of 48 oz. } *assorted sizes of 7020, 7022 and 7024, No. 1 and No. 2*
20% discount in lots of 72 oz. }

7024. Micro Cover Glasses, Rectangles, Non-Corrosive, Red Label, as above described. Packed in ½ oz. pasteboard cartons.

Size, mm	22 × 30	22 × 32	22 × 36	22 × 40	22 × 50	24 × 30	24 × 36	24 × 40
No. 1, per oz.	**1.50**	**1.50**	**1.50**	**1.50**	**1.50**	**1.50**	**1.50**	**1.50**
Code Word	*Juoyq*	*Jupci*	*Jupju*	*Jupoj*	*Jupsb*	*Jupyp*	*Juqiv*	*Juqyo*
No. 2, per oz.	**1.30**	**1.30**	**1.30**	**1.30**	**1.30**	**1.30**	**1.30**	**1.30**
Code Word	*Jusml*	*Juspe*	*Juszo*	*Jutai*	*Jutfy*	*Jutko*	*Jutrz*	*Jutyl*
Size, mm	24 × 50	24 × 60	35 × 50	35 × 62	43 × 50	43 × 70	48 × 60	
No. 1, per oz.	**2.45**	**2.45**	**2.45**	**2.45**	**2.45**	**2.45**	**2.45**	
Code Word	*Jurde*	*Jurfa*	*Jurgy*	*Jurlo*	*Jurpf*	*Juruv*	*Jusaj*	
No. 2, per oz.	**2.00**	**2.00**	**2.00**	**2.00**	**2.00**	**2.00**	**2.00**	
Code Word	*Juucd*	*Juudb*	*Juufx*	*Juuir*	*Juumj*	*Juuqa*	*Juury*	

10% discount in carton of 6 oz., one size and thickness of 7024 only
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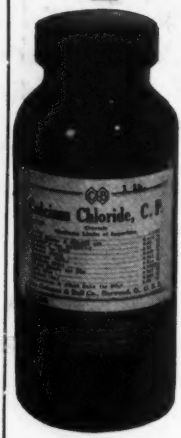

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